Doping in sports
The effect of alcohol on the urinary increase of testosterone I epitestosterone

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DOPING IN SPORTS: THE EFFECT OF ALCOHOL ON THE URINARY INCREASE OF TESTOSTERONE/EPITESTOSTERONE

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SUBMITTED FOR PH.D

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“Só é vencido quem desiste de lutar.”

‘The one who gets defeated is the one who stops fighting.’

Abstract

Testosterone (T) administration is prohibited in sport by the World Anti-Doping Agency (WADA). Following T administration, both T concentration and the ratio to its inactive stereoisomer, epitestosterone (E), increases. WADA recommends that their accredited laboratories undertake further analysis on urine samples with a T/E greater than 4.0. Acute alcohol consumption (2.0 g/kg) appears to increase this ratio but there is little supporting published data.

This thesis describes the testing of different hypotheses based on the influence of alcohol administration on the T/E ratio and attempts to establish the underlying mechanisms.

Alcohol was administered to eugonadal men and women (4 and 8 units), and also to hypogonadal men (8 units) receiving T replacement therapy. Hypogonadal men were selected as changes in T would be due solely to altered clearance rather than gonadal production, the data obtained making a useful comparator model.

Following alcohol administration, the urinary T/E increased significantly in all the volunteers, in women (8 units) rising from approximately unity to greater than the WADA threshold.

Serum analysis showed a decrease in T in males, which indicates that the increase in urinary T/E is most likely due to an increase in the hepatic formation of T glucuronide, which is rapidly excreted, not to an increase in gonadal production. The increase in serum T in females will most likely be due to a suppression of phase 1 metabolism of T, via enzyme inhibition (17β-HSD2), which is likely to also occur in men but is masked by the proportionately much larger concentration of circulating T.

There is no evidence that EtOH displaces T from its binding proteins increasing T clearance with a resulting increase in urinary T/E, as tested *in vitro* by equilibrium dialysis and ultrafiltration, nor is there any evidence that an increase in T/E is due to an increase in lutenising hormone.

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Sponsor

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Abbreviations

AAI	Acute Alcohol Intoxication
ABV	alcohol by volume
ACTH	adrenocorticotropic hormone
AD	Androstenedione
ADH	alcohol dehydrogenase
ALDH	acetaldehyde dehydrogenase
AVP	arginine vasopressin
BAC	blood alcohol concentration
BNST	bed nucleus of the stria terminalis
CBG	corticosteroid binding globulin
CRH	corticotropin-releasing hormone
DHEA	dehydroepiandrosterone
DHT	5α – dihydrotestosterone
DOC	deoxycorticosterone
E	epitestosterone
E2	estradiol
EtG	Ethyl glucuronide
EtS	Ethyl sulfate
FSH	follicle stimulating hormone
g	gram
GC-MS	gas chromatography-mass spectrometry
GC-C-IRMS	gas chromatography carbon ion ratio mass spectrometry
GnRH	gonadotropin releasing hormone
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
IA	immunoassay
IOC	International Olympic Committee

kDa	kilo Dalton
LH	luteinizing hormone
LLOQ	lower limit of quantification
MCR	Metabolic clearance rate
mg	milligram
min	minutes
mL	millilitre
MS	mass spectrometer
MSTFA	N- methyl-N-trimethylsilyl-trifluoroacetamide
MW	molecular weight
NF	normal females
NM	normal males
PBS	Phosphate buffer saline
PR	production rate
PVN	paraventricular nuclei
S/N	signal to noite
SD	standard deviation
SHBG	sex-hormone binding protein
SPE	solid phase extraction
SRM	selected ion monitoring
T	testosterone
T/E	testosterone to epitestosterone ratio
TBC	testosterone's binding capacity
TBG	thyroid-binding globulin
TMS	trimethylsilyl
WADA	World Anti-Doping Agency

Chapter 1 INTRODUCTION

1.1 Doping in sports and forbidden substance

1.1.1 Foreign (xenobiotics) and pseudoendogenous substances

The will to succeed in competitive sports is such that an athlete may use performance enhancing substances to help him/her go the extra-mile. Many of these substances may be harmful to the athlete and/or raise ethical issues with regard to what is considered a 'fair competition' and are thus prohibited within this setting. The practice of using prohibited drugs is often referred to as 'doping', which is defined in the Oxford English Dictionary as the "act of taking a drug to improve the performance of an athlete, racehorse or greyhound".

The financial, personal and national prestige can put pressure on an athlete to succeed, and therefore taking substances that will increase his/her performance may seem like an attractive option (Figure 1.1).

Figure 1.1 Doping in sport (Ariens, 1965)

The death of the Danish cyclist Knud Enemark Jensen linked to the use of amphetamines during the Olympic Games of 1960 led to drastic changes to the rules and regulations of sporting events. The sporting community recognised that some form of control was needed with regard to the use of performance enhancing substances to prevent future tragedies (World Anti-Doping Agency, 2009a).

In 1967, the International Olympic Committee (IOC) compiled a list of forbidden substances and anti-doping tests took place in the Winter Olympic Games in Grenoble the following year. Since then, the list of banned substances and classes of drugs they fall under has grown.

In some countries, such as France, doping is a criminal offence. In 1998, a police raid performed during the Tour de France apprehended a large amount of prohibited substances. Since doping sanctions were sometimes overruled in criminal courts, there was a need to create an international, independent organization that would produce a harmonised set of anti-doping standards, as well as work with sports associations and public authorities. The World Anti-Doping Agency (WADA) was therefore created in 1999 after a world conference on “Doping in Sports”.

One of the WADA’s main accomplishments is the creation of an international World Anti-Doping Code, which brings together anti-doping policies, rules and regulations for sports associations and guidelines on co-ordination between anti-doping organizations, testing laboratories, therapeutic use exemptions, the list of prohibited substances and analytical methods (World Anti-Doping Agency, 2009b).

The Prohibited List is published every year and it contains the substances and methods prohibited at all times, both in- and out-of-competition. Section S1 of the Prohibited List comprises the banned anabolic androgenic steroids including testosterone (T) (17β-hydroxy-androst-4-ene-3-one) and its isomer epitestosterone (E) (17α-hydroxy-androst-4-ene-3-one) (Figure 1.2). These substances are prohibited at all times (in- and out-of-competition). An approved test for the detection of testosterone administration is based on a urinary ratio (peak height) of testosterone to epitestosterone (T/E>4), above which WADA accredited laboratories are required to report an atypical finding.

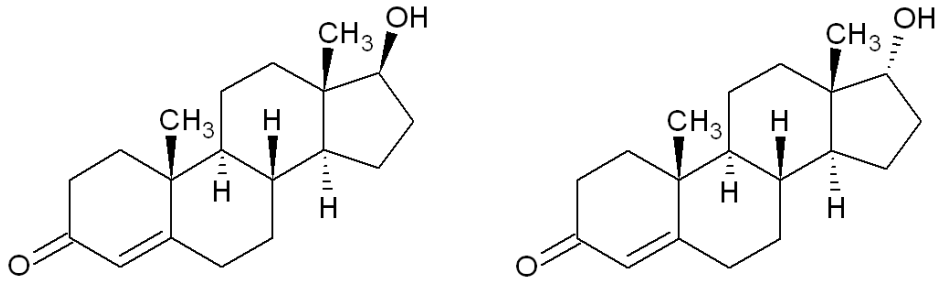


Figure 1.2 Testosterone & epitestosterone

1.2 The endocrine system – gonadal and adrenal hormones

1.2.1 The endocrine system

The endocrine system, the nervous system and the immune system regulate the human body. They help maintain the body in a state of internal homeostasis altering it if necessary in response to external changes (Greenstein, 1994a).

The endocrine system comprises a number of glands which secrete hormones into the blood stream. Hormones are *"chemical substances that are produced by a specialised ductless gland in one part of the body and secreted directly into the blood stream and then carried to a distant organ where a regulatory response or action is elicited, e.g. cortisol secreted by the adrenal gland, testosterone by the testis, estradiol by the ovary"* (Kicman, 2007)

This system regulates most metabolic activities, and the rate of most endocrine secretions (hormones) is activated by the brain.

Hormones are chemical messengers that control and co-ordinate organ and tissue function. They are secreted as a result of nerve impulses that are triggered by physical or psychological stress (Emanuele and Emanuele, 1997). Sensitive feedback mechanisms reduce or increase the amount of hormones released into the blood stream.

A feedback mechanism is a closed-loop system where the returning signal results in a reduction or increase in system output (Reichlin, 1992). A negative feedback mechanism responds by reducing the output of the hormone whereas positive feedback responds by increasing the hormone.

A list of the principal endocrine glands and their corresponding secreting hormones can be found in Appendix 8.1.

For glands present in the human body, please see Figure 1.3.

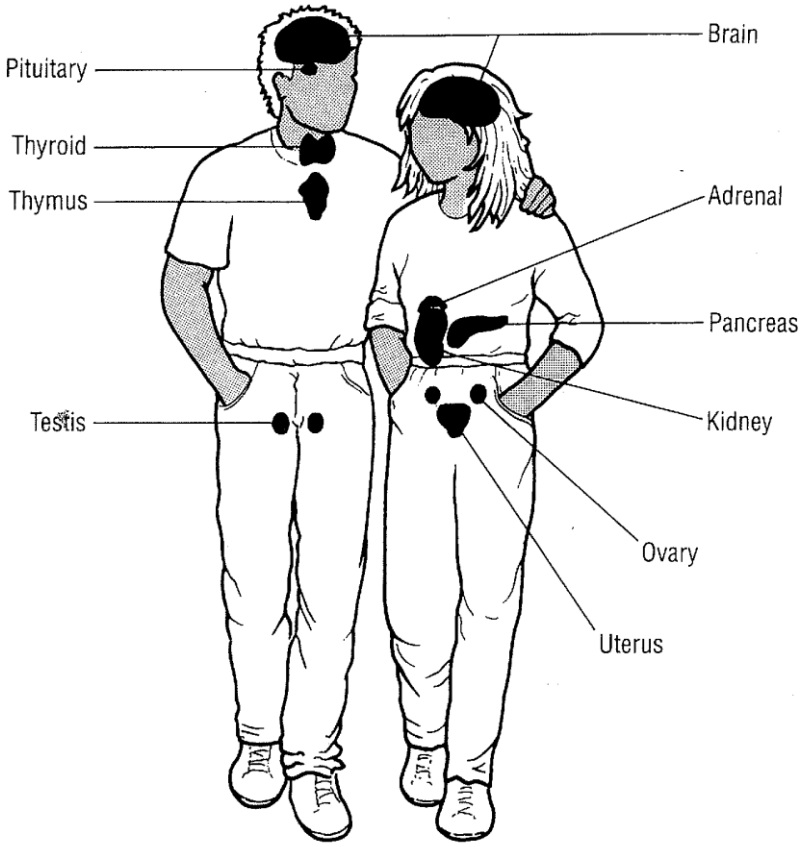


Figure 1.3 Location of endocrine glands (Greenstein, 1994a)

The hypothalamus and the pituitary gland regulate much of the endocrine system and these glands are intimately associated.

Figure 1.4 shows a diagram of the human brain to illustrate the location of the hypothalamus and pituitary gland.

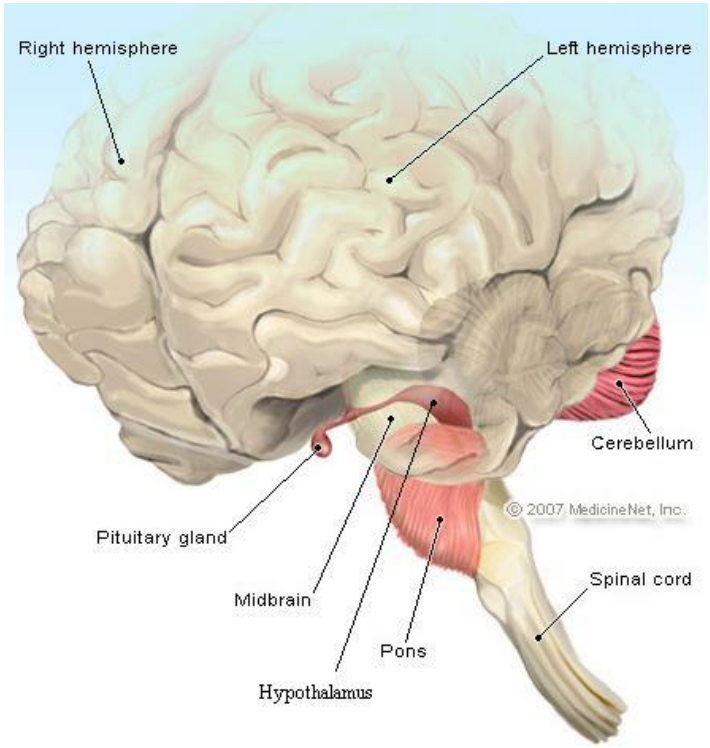


Figure 1.4 Modified brain map (E-MedicineHealth, 2007)

The pituitary gland is divided into three lobes:

- Anterior lobe (also called the anterior pituitary, adenohypophysis, pars distalis or pars glandularis)
- Posterior lobe (also called the neural lobe or infundibular)
- Intermediate (also called the pars intermediate)

The intermediate lobe is not found in adult humans since, although we are born with it, it is distributed evenly by the anterior and posterior lobes (Reichlin, 1992) during the first two decades of life (Figure 1.5).

Pituitary secretion of hormones is dependent upon hypothalamic secretion of the corresponding releasing hormone, and the hypothalamus selectively secretes the appropriate releasing hormone to control the concentration of the related hormone as required.

The hypothalamus secretes its releasing hormones into the anterior pituitary via the funnel-like hypothalamic-hypophyseal portal veins (Figure 1.6). These connect the hypothalamus to the anterior pituitary via a branch of blood capillaries in the lower hypothalamus, that are in their turn connected to a neural stalk, the neural lobe and another capillary bed within the anterior pituitary. These capillaries also carry hormones secreted by the anterior pituitary gland, such as luteinising hormone, follicle-stimulating hormone, thyroid stimulating hormone and growth hormone, and end-up combining with blood vessels that will transport these hormones into the systemic venous blood. These veins also collect capillary blood from the posterior pituitary gland (Bowen, 2001, Reichlin, 1992, Constanti and Bartke, 1998).

The posterior pituitary hormones, arginine vasopressin and oxytocin, are produced in the supraoptic and paraventricular nuclei of the hypothalamus, respectively (Porterfield, 2001b).

These are transported to the posterior pituitary via the nerve endings in the hypothalamus.

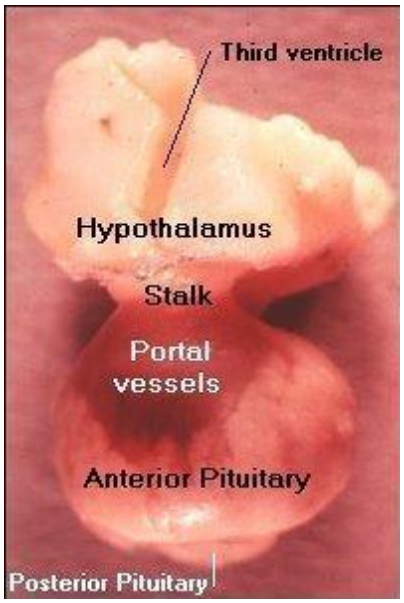


Figure 1.5 Pituitary gland (Bowen, 2001)

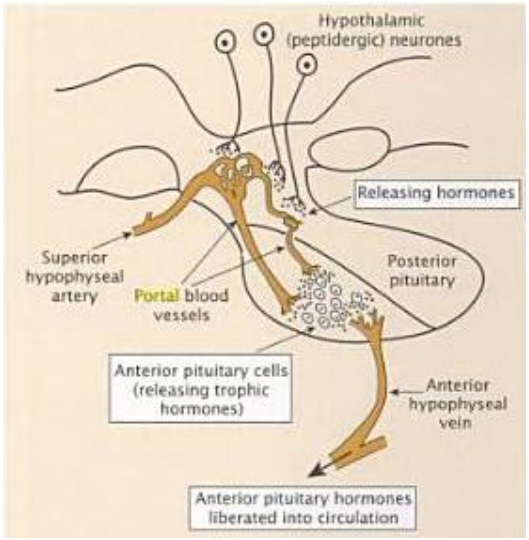


Figure 1.6 Hypothalamic-hypophyseal portal system (Constanti and Bartke, 1998)

1.2.2 Hypothalamic-Pituitary-Gonadal axis

The hypothalamic-pituitary-gonadal (HPG) axis comprises the hypothalamus, the anterior pituitary gland and the gonads (testes for men and ovaries for women), and is responsible for the secretion of androgens.

Androgens are steroid hormones secreted by the gonads and the adrenal cortex. Androgens have their effect in reproductive tissues, muscle, bone, hair follicles in the skin, liver and kidneys, the immune and the central nervous system (Kicman, 2008). The effects of these hormones mainly linked to masculinisation and anabolic effects such as protein building in skeletal muscle and bone. In males, androgens are responsible for the development and maintenance of the male accessory reproductive glands, prostate and seminal vesicles, size of the testes, scrotum and penis (Gower, 1984a). The secondary sexual characteristics during puberty may be divided into what results from androgenic and anabolic effects. The androgenic effects are responsible for the enlargement of the larynx which causes deepening of the voice, the growth of terminal hair (pubic, axillary and facial regions), an increase in sebaceous gland activity (which may lead to acne), and central nervous system effects (increased libido and aggression). The anabolic effects are responsible for growth of skeletal muscle and bone (Kicman, 2008).

Testosterone is the most important androgen secreted, and it is regulated by a negative feedback mechanism in which the hypothalamus releases the gonadotropin releasing hormone (GnRH), also called luteinizing hormone releasing hormone (LHRH), that stimulates the anterior pituitary gland to release the two gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the blood stream (Emanuele and Emanuele, 1997).

In men, when LH reaches the testes it stimulates T synthesis in the Leydig cells whereas FSH alongside with T stimulates the Sertoli cells prompting spermatogenesis. The circulating T will then return to the hypothalamus and control the secretion of GnRH, and consequently the secretion of LH and FSH by the anterior pituitary gland (Griffin and Wilson, 1992) (Figure 1.7). In summary, in this closed loop system (hypothalamus-anterior pituitary gland-testes), the returning signal (T) results in a reduction in system output (LH and FSH).

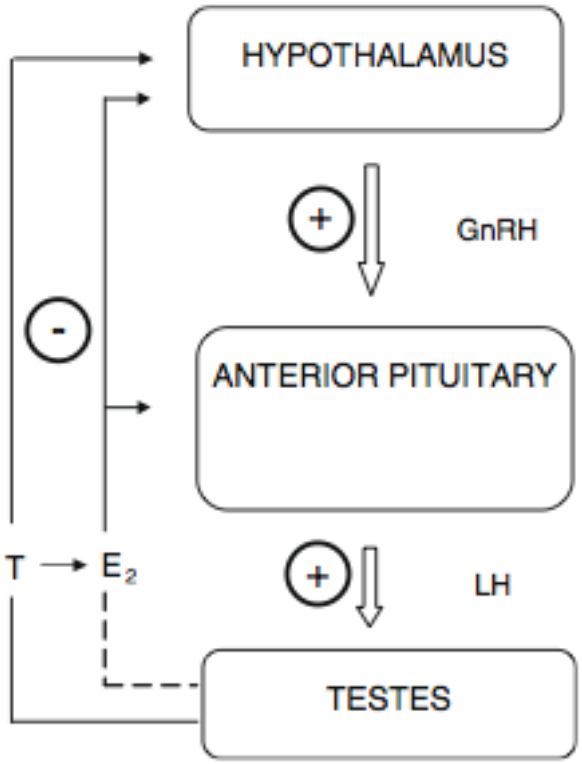


Figure 1.7 Negative feedback mechanism in men where the (+) indicates excitement and (-) inhibition (Kicman, 2010)

Radioimmunoassay studies have shown that the release of GnRH is episodic, occurring approximately every 90 min (Brook and Marshall, 2001c, Greenstein, 1994d) into the hypothalamic-hypophyseal portal veins described earlier. GnRH binds to its receptor in the anterior pituitary gland, causing the pulsatile release of LH and FSH every 90 min in response to pulses of GnRH (Brook and Marshall, 2001a).

The contributions of T and estradiol (E2) to LH secretion and the importance of E_2's aromatization in T's effects in the HPA axis were debateable, but the study by Pitteloud *et al,* showed that T and E_2 have independent effects in LH secretion (Pitteloud et al., 2008).

This study was performed in eugonadal men and men with idiophatic hypogonadotropic hypogonadism who are GnRH deficient, during 7 days (n=22 for both). These men were on a hormone replacement therapy to normalize their hypothalamic-pituitary axis. Since the dose and frequency of GnRH was controlled, any effect on consequential gonadal steroids by GnRH could only come from the pituitary.

In both groups, ketoconazole was administered in high doses to produce a biochemical castration. Ketoconazole is a potent inhibitor of C_{17-20} lyase (rate-limiting step in androgen biosynthesis) that suppresses T to castrate levels in 24 h. In high doses (1 mg/day) it also inhibits cortisol's biosynthesis and aromatase activity (Weber et al., 1991, Wouters et al., 1988). Subsequent sex-steroid replacement was employed. Both groups were administered with T and E2 independently, and their concentrations were monitored together with LH.

In eugonadal men, following T administration, there was a decrease in GnRH's pulse frequency while E2 levels were suppressed indicating that T feedback in the hypothalamus could occur without aromatization but by direct androgen effect. By contrast, when T was administered to hypogonadal men, the LH concentration did not decrease thus indicating that T's negative feedback mechanism in the pituitary is mediated by aromatization to E2.

After E2 was administered in eugonadal men, there was a decrease in GnRH pulse frequency thus confirming a hypothalamic effect of E2. In the hypogonadal group of volunteers, after E2 intake, the concentration and pulse amplitude of LH decreased. Since this group does not produce GnRH, the LH suppression is indicative of a pituitary site of negative feedback mechanism.

In women, both LH and FSH release are stimulated by GnRH, but the negative feedback mechanism is inhibited by E2 and progesterone (Figure 1.8). Progesterone is synthesised from pregnenolone which in turn comes from cholesterol (for a detailed synthesis please see Figure 1.11 and Figure 1.15). Progesterone concentrations in normal females range from 0.2-1.2 ng/mL (0.6-3.8 nM) in the follicular phase, 0.72-17.8 ng/mL (2.3-56.6 nM) in luteal phase, 0.2-1 ng/mL (0.6-3 nM) in postmenopausal women and 0.34-0.92 ng/mL (1.1-2.9 nM) in women taking oral contraceptives. In men over 16 years-old, progesterone concentration range from 0.27-0.9 ng/mL (0.86-2.9 nM) (National Institute of Health, 2009, Stricker et al., 2006, D.B. Gower and J.W. Honour, 1984).

In women, progesterone is produced in the ovaries, before ovulation by influence of LH, and during pregnancy in the placenta. It is responsible for preparing the ovaries for a fertilized ovum, and if the ovum is not fertilized then progesterone levels decrease and menstruation occurs.

The biosynthesis of progesterone occurs from pregnenolone via 3β–HSD enzyme, with cholesterol being the precursor of pregnenolone (Figure 1.15) (Wood and Gower, 2010).

It has also been suggested to use serum 17α-hydroxyprogesterone's (17-OHP) ratio with T (T/17OHP) as a marker to monitor exogenous T intake for doping purposes, in studies performed in 7 males (Carlstrom et al., 1992, Palonek et al., 1995) and 11 males (Palonek et al., 1995). One of the precursors of testicular T is 17OHP with 75 % of it coming from testicular synthesis (Crilly et al., 1981). Administration of synthetic anabolic steroids suppresses 17-OHP concentrations by ~ 75 % (Alen and Hakkinen, 1987) and in an increased serum T/17-OHP could be used as an additional marker.

However, the use of T/17OHP is not described in the WADA's technical document for T's detection (World Anti-Doping Agency, 2004). This may be due to the fact that there are already two ancillary methods such as urinary T/LH and urinary androsterone/testosterone (A/T) put in place thus serum T/17OHP becomes obsolete; or perhaps because conversion of 17OHP into T follows Δ^4 pathway from pregnenolone which is a minor route in males. The most common biosynthetic pathway for T in humans is via Δ^5 pathway from pregnenolone (for more details on biosynthesis of T please see section 1.3.2).

LH will act on the ovaries to initiate steroidogenesis in the ovarian follicle, causing ovulation and maintenance of the ovulatory functions of the corpus luteum. FSH will stimulate the development of the ovarian follicles and their secretion of E2.

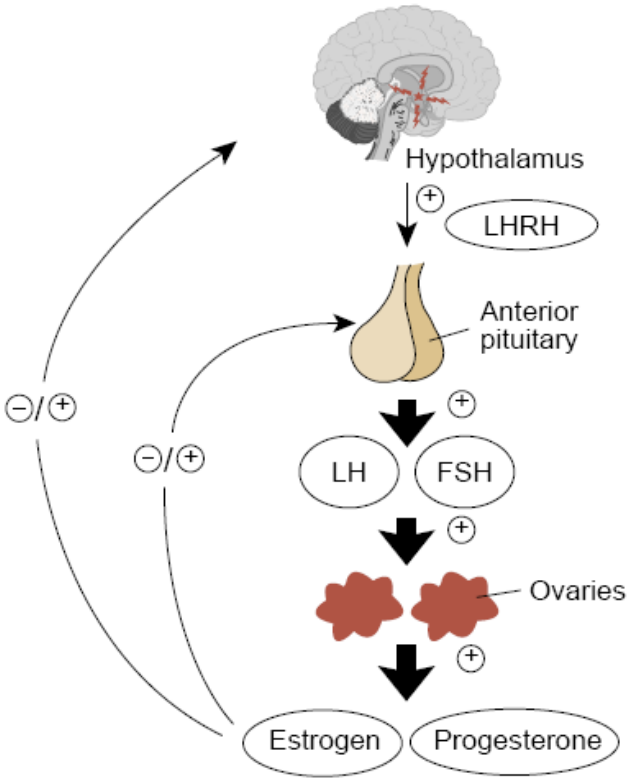


Figure 1.8 Negative feedback mechanism in women (Emanuele and Emanuele, 1997)

The only situation in which LH is not controlled via a negative feedback mechanism happens just before ovulation where high concentrations of estrogens are associated with a sudden rise in LH (Brook and Marshall, 2001a) (Yen and Lein, 1976).

In men, as mentioned previously if the system is working normally then a lowering of the circulating T concentration will result in a rise in GnRH and consequently LH and FSH and *vice versa* (Emanuele and Emanuele, 2001, Griffin and Wilson, 1992).

This is valid only for males, since LH is suppressed in females with oral contraception. The urinary concentration in healthy men (n=120) is approximately 0.5-20 IU/L (international units per litre) (Drug Control Centre, 2010).

In contrast, an increase in the circulating concentration of T will suppress LH, and this is why LH measurements are useful in sports. Doping with T results in a lower circulating concentration of LH, reflected in a higher excretion of T and a lower excretion of LH, and samples with a high urinary T/LH ratio are considered suspect. Analysis of healthy male urine (n=120) has shown

that the reference interval for urinary T/LH ratio should be between 1-150 nmol/IU (Drug Control Centre, 2010).

The reference range for LH in serum is 5-20 IU/L (Griffin and Wilson, 1980). A previous study, that analysed LH during daytime from 8 hours (h) to 14 h, and at night time from 20 h to 2 h, showed no difference in the amount secreted in these two periods of time (n=12), nor did it show any significant difference in the half-life between sexes (Clark et al., 1997).

The metabolic clearance rate[1] (MCR) of LH is 24 mL/min in men (Veldhuis et al., 1986), and in post-menopausal women is 0.14 mL/min (Keller, 1966).

After both sexes reach puberty the secretion of LH increases, causing a maturation of the Leydig cells and consequent secretion of T. Whereas in women the increase of T although significant is smaller than in men partly because about half originates from secretion by the ovaries and the adrenal cortex, and the other half from peripheral conversion of androstenedione (AD) (Wheeler, 1995, Horton and Tait, 1966).

1.2.3 Hypothalamic-Pituitary-Adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis, like the HPG axis described above, is controlled via a negative feedback mechanism that comprises the hypothalamus, the anterior pituitary gland and the adrenal glands.

The HPA axis is also regulated by the hypocampus, amygdala, bed nucleus of the stria terminalis (BNST) and paraventricular nuclei (PVN).

This axis mediates the response to stress levels, so situations of physical or emotional stress activate the HPA axis.

The adrenal gland is situated above the kidneys and can be divided into two sub-organs: the adrenal cortex, which secretes steroid hormones, and the adrenal medulla that secretes catecholamines such as adrenaline and noradrenaline that increase serum glucose and fatty acid levels (Greenstein, 1994b).

[1] Volume of biological fluid completely cleared of substance in unit time.

The adrenal cortex is divided into three zones:

- Zona glomerulosa (synthesizes aldosterone)
- Zona fasciculata (synthesizes cortisol)
- Zona reticularis (synthesizes dehydroepiandrosterone (DHEA) and AD)

For the purpose of this review only steroid hormones secreted by the adrenal cortex are of relevance. These are mineralocorticoids, glucocorticoids and sex hormones.

Mineralocorticoids regulate mineral metabolism, and the two most potent ones are aldosterone and deoxycorticosterone (DOC). Aldosterone also has some mild glucocorticoid activity. Glucocorticoids regulate blood glucose, have potent anti-inflammatory and immunosuppressive properties and are used to treat arthritis and dermatitis. Their use is prohibited in sports; the most potent one is cortisol, which also has some mineralocorticoid activity (Porterfield, 2001c).

The PVN cells in the hypothalamus secrete 2 hormones: corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP). These act on the pituitary gland via the hypothalamic-hypophyseal portal system. When CRH reaches the anterior pituitary gland, it will bind to the specific receptors – the corticotropes – that in their turn will secrete adrenocorticotropic hormone (ACTH) (Emanuele and Emanuele, 1997).

CRH is the primary regulator of stress response and, if CRH is stimulated, secretion of ACTH will increase. ACTH is then carried in the blood to the adrenal cortex and, in its turn, interacts with the receptors on the adrenocortical cells that stimulate the production and release of cortisol (Figure 1.9) (Varghese et al., 2001).

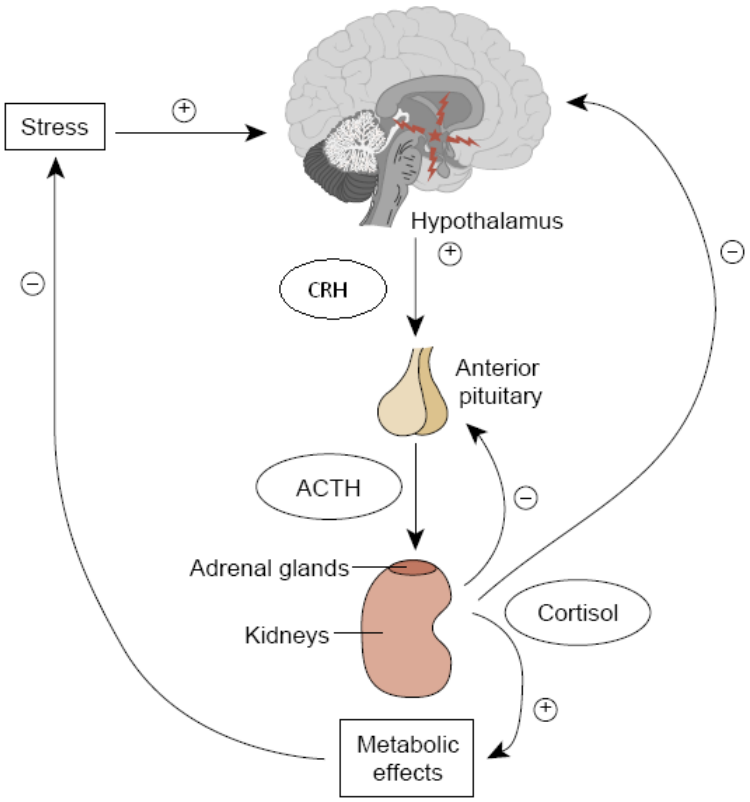


Figure 1.9 Negative feedback mechanism from HPA axis (Emanuele and Emanuele, 1997)

Cortisol (Figure 1.10) is the main glucocorticoid in humans and is interconverted to cortisone (another glucocorticoid) in the liver. It triggers metabolic effects with the purpose of relieving stress states in the body. The adrenal gland also produces corticosterone, which mainly has mineralocorticoid activity but some glucocorticoid one as well.

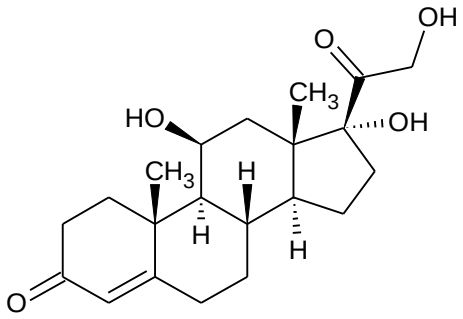


Figure 1.10 Cortisol

Cortisol exerts a negative feedback at the level of the hypthalamus and pituitary glands similar to that of the HPG axis described earlier. Stress increases secretion of CRH by the hypothalamus, which in turn increases ACTH by the anterior pituitary, thus releasing cortisol by the adrenal glands (Porterfield, 2001c).

Both ACTH and cortisol have a diurnal variation, which is a function of the diurnal secretion of CRH. Secretion of ACTH has its peak in the early morning just before sunrise and slowly decreases throughout the day (Porterfield, 2001c). The main sex hormones secreted by the adrenal glands are AD and DHEA. These weak androgens can be converted into more potent ones, like T, or into estrogens in peripheral tissues. (Porterfield, 2001c) (Figure 1.11). This is of greater relevance in females than in males due to the small amount of T produced in females (Kicman, 2010).

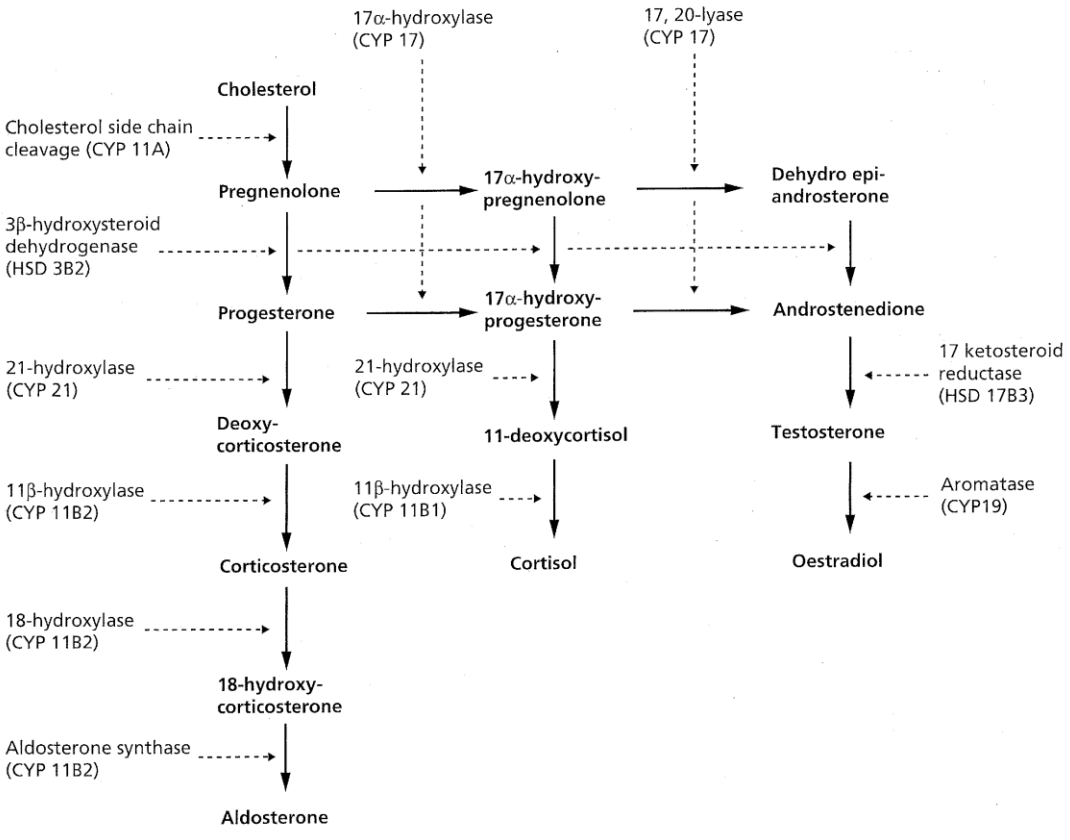


Figure 1.11 Synthesis of adrenal steroid hormones (Brook and Marshall, 2001b)

1.3 Androgens

Androgens are sex steroids, primarily responsible for the maintenance and function of the prostate and seminal vesicles, particularly with spermatogenesis, and cause an increase in the testes size, scrotum, penis, seminal vesicles and prostate, (Brooks, 1975b, Gower, 1979). Testosterone is one of the most biologically active androgens, together with 5α-dihydrotestosterone (5α-DHT), both having a 17β-hydroxyl group and a 3-oxo group.

Androgens are secondarily responsible for protein muscle increase due to their anabolic effects, and finally they also have an effect on non-sexual organs and tissues such as the growth of sexual, axillary and facial hair throughout puberty, deepening of the voice in boys, and an increase in the activity of sebaceous glands resulting in acne in most cases (Brooks, 1975b, Gower, 1979, Gower, 1984a). The steroids T, 5α-DHT and 5α-androstane-3β,17β-diol have higher androgenic activity. Oxidation of the 17β-hydroxyl group or reduction of the 3-oxo group lead to loss in androgenic activity (AD and 5α-androstenediol), and when both happen in the steroid molecule it leads to an even weaker androgenic effect like in DHEA and androsterone. Etiocholanolone and E have no androgenic activity.

The changes in the 17β-hydroxyl and/or 3-oxo groups compared to T and 5α-DHT may be found in bold in Figure 1.12.

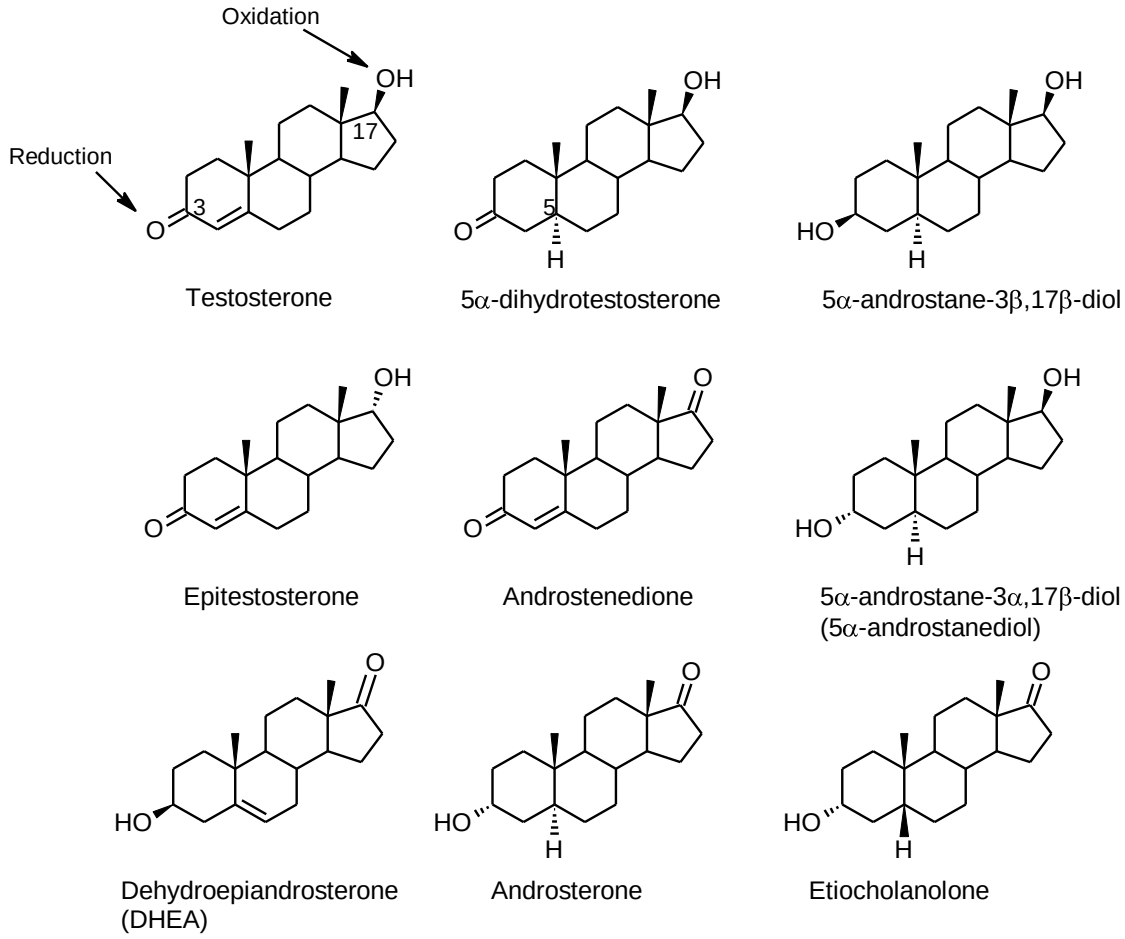


Figure 1.12 Structure of C_{19} endogenous androgens based on (Kicman, 2010)

1.3.1 IUPAC nomenclature

A steroid is a molecule that has the "*skeleton of cyclopentanophenanthrene or a skeleton derived therefrom by one or more bond scissions or ring expansions or contractions"* (International Union of Pure and Applied Chemistry, 2009).

Steroids are four fused ringed compounds that originate from cholesterol in the form of cholesterol esters, lipoproteins or *de novo* synthesis. Each steroid is named using the carbocycle as the main structure: estrane (C_{18}), androstane (C_{19}) or pregnane (C_{21}) as seen on Figure 1.13.

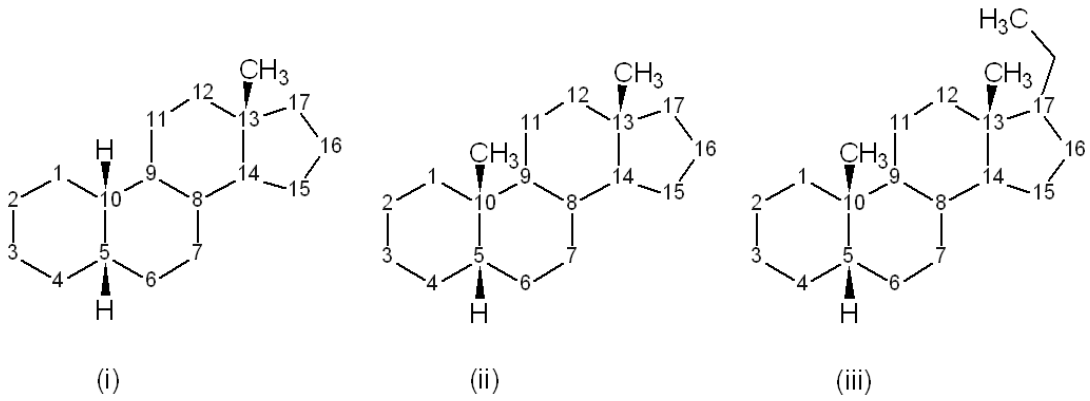


Figure 1.13 5β-estrane (i), 5β-androstane (ii) and 5β-pregnane (iii)

The carbon numbering of steroids in the cholestane molecule is shown in Figure 1.14.

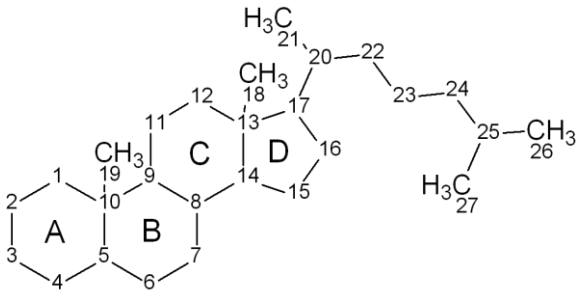


Figure 1.14 Labelling of a steroid molecule (cholestane)

1.3.2 Biosynthesis and production of testosterone

Biosynthesis of T occurs mainly in the testes (Leydig cells) and the adrenal cortex, and to a lesser extent in the ovaries and placenta.

Serum concentration for eugonadal males is usually 3 – 10 ng/mL (10 – 35 nmol/L) and for females less than 1 ng/mL (3.5 nmol/L).

In women, T is produced in the ovaries to a lesser extent and the peripheral conversion of androgens is the major contributor to T production. The androgens that the adrenal cortex mainly secretes are AD, DHEA and DHEA sulfate (Weigel and Moore, 2007b, Mendelson and Stein, 1966). There is peripheral interconversion between DHEA sulfate and DHEA (Kakihana et al., 1971), DHEA is peripherally interconverted to AD, and there is peripheral interconversion

between AD and T. This peripheral conversion, although happening to an extent of 12 – 14%, accounts for half of the circulating T in women (Bardin and Lipsett, 1967b).

There are two synthetic pathways for T's biosynthesis: one via 5-ene-3β-hydroxysteroids such as 17-hydroxypregnenolone and DHEA (Δ^5 pathway); and the 4-en-3-oxosteroids such as progesterone and 4-AD (the Δ^4 pathway) (Figure 1.15).

The Δ^5 pathway is the most common in males (Ruokonen et al., 1972) and the path shown in bulky arrows is the main route in humans.

The enzymes involved in this biosynthesis are cytochrome P17 (CYP17) and 17β – hydroxysteroid dehydrogenases (17β – HSD) (Payne and Hales, 2004).

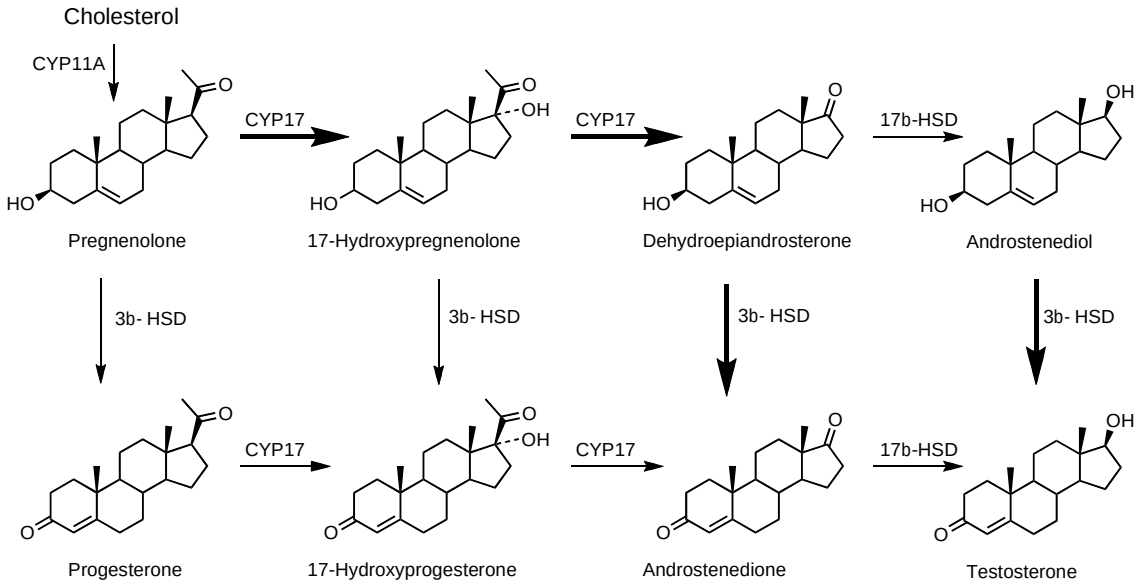


Figure 1.15 Biosynthethic pathway of T with respective enzymes involved. The first pathway is Δ^5 and the second Δ^4 (Payne and Hales, 2004, Slaunwhi.Wr and Burgett, 1965, Kicman, 2010)

Pregnenolone and progesterone are hydroxylated at carbon 17, followed by side chain cleavage, catalysed by CYP17 and resulting in C_{19} steroids, DHEA and AD.

Conversion of 17-hydroxypregnenolone into DHEA by side chain cleavage was shown by studies in rats (Slaunwhi.Wr and Burgett, 1965). After the addition of 17-hydroxypregnenolone,

no noticeable increase in AD was verified for the first 15 mins, suggesting that this oxidation was not happening to a high extent. However, at the same time T was being formed rapidly, showing that the reduction from DHEA to androstenediol, and subsequently the oxidation from androstenediol to T, were occurring at a fast rate.

These results suggested that there was an alternative pathway from 17-hydroxypregnenolone to androstenediol, without involving DHEA to a great extent since this is rapidly converted to AD. It was suggested that AD was biosynthesized by the Δ^4 pathway and T by the Δ^5.

The Δ^5 pathway originates 17-hydroxypregnenolone, DHEA, androstenediol and T; whereas the Δ^4 pathway originates 17-hydroxyprogesterone, AD and T.

Work on incubated mouse and rat testes with ^{14}C-labelled sodium acetate, found radioactivity associated with cholesterol and T, and also to AD but to lesser extent (Delatorre et al., 1977). No labelled C_{21} steroid (pregnenolone, progesterone and their 17-hydroxylated derivatives) or DHEA (C_{19} steroid) was found. This indicated that, if DHEA was formed directly from cholesterol, it must have been transformed into AD and the majority of it reduced to T by the 17-hydroxysteroid dehydrogenase.

This study contrasts with human testicular preparations, which were also incubated under the same conditions as above but resulted in the formation of AD and T and of some (or all) C_{21} and C_{19} steroids intermediates expected.

The enzymes involved in T's biosynthesis can be divided into two categories of protein: the cytochrome P450 haeme-containing proteins, or the hydroxysteroid dehydrogenases (Payne and Hales, 2004).

The first family of enzymes has the nomenclature of CYP for human genes (Cyp for animals) followed by a number indicative of the P450 family in which the enzyme can be found. In cases of existing subfamilies, the numbers are followed by a capital letter (eg. CYP11A, enzyme responsible for the synthesis of cholesterol to pregnenolone).

These enzymes are responsible for the hydroxylation and cleavage of the steroid substrate in the biosynthesis of steroid hormones from cholesterol. They act as mono-oxygenases utilizing

reduced nicotinamide adenine dinucleotide phosphate (NADPH) as the electron donor for the reduction of molecular oxygen (Payne and Hales, 2004). The oxygen (O_2) is activated by P450 and while one atom is introduced into the RH substrate as an hydroxyl group, the other atom is reduced to H_2O:

$$RH + O_2 + NADPH + H^+ \longrightarrow ROH + H_2O + NADP^+$$

The second family of enzymes (hydroxysteroid dehydrogenases) includes the 3βHSDs and the 17βHSDs. These enzymes are oxidoreductases and are involved in the reduction and oxidation of steroid hormones requiring the cofactors nicotinamide adenine dinucleotide (NAD(H)) and its phosphate (NADP(H)). The forms NAD^+/$NADP^+$ work as acceptors and their reduced forms as donors.

It is the oxidised form of the cofactors that work in the catalysis as hydrogen acceptor and the reduced species as a donor:

$$NAD^+ + 2H^+ \rightleftharpoons NADH + H^+$$

17β-HSD3 has a reductive primary activity converting AD to T, preferring NADPH as a cofactor.

17β-HSD reactions are catalyzed by multiple isozymes that favour, in general, the reduction of the biologically inactive 17-ketosteroids to active 17β-hydroxysteroids in the gonads, and the reverse oxidative reaction in peripheral tissues.

The main difference between this family of enzymes and the P450 one, is that the first has several isoforms (several different forms of the same protein) for 3βHSDs and several isoenzymes (enzymes that differ in amino acid sequence but catalyze the same chemical reaction) for the 17βHSDs, whereas the last family of enzymes is the product of a single gene.

1.3.3 Biosynthesis of epitestosterone

Epitestosterone is an epimer of T, and a reference steroid for T's abuse for doping purposes, excreted mainly as a glucuronide.

Its concentration in serum is age dependent, reaching its peak in men at 35 years old and in women at 20 years old, whereas the average concentration in an adult men averages 2.5 nmol/L (0.72 ng/mL) and 1.2 nmol/L (0.34 ng/mL) in women (Havlikova et al., 2002). As for the urinary excretion of E, the average for normal males is 166.4 (range 8.7-396) μg/24 h, and for normal females 11.1 (range 6.5 – 19.6) μg/24 h (Denicola et al., 1966), or 578 (range 30 – 1375) nmol/24 h for males and 39 (range 23 – 68) nmol/24 h.

In humans, a small part is converted from AD or DHEA (Brooks and Giuliani, 1964) and peripheral metabolism from T to E is negligible (Dray and Ledru, 1966, Starka, 2003).

One of the suggested pathways for E's synthesis is from the oxidation of epiandrostenediol (androst-5-ene-3β, 17α-diol) by 3βHSD since this diol was found in human testis (Ruokonen et al., 1972, Starka, 2003). Its correlation with concentrations of epitestosterone in the spermatic vein has been documented (Dehennin, 1993), also showing the secretion of E by the human testes. The testes are the main source of E secretion, with a modest contribution from the adrenal gland (Kicman et al., 1999).

The other one is via the synthesis of AD, shown by (Catlin et al., 2002) that by orally administering AD to healthy male volunteers provoked an increase in urinary excreted E via 17βHSD, confirmed in human embryonic kidney cells by Bellemare *et al.* (Bellemare et al., 2005).

The efficiency of 3βHSD for DHEA and of 17βHSD for AD shows that the main pathway is from conversion of DHEA to AD and then reduction to E, and the minor pathway from the reduction of DHEA to epiandrostenediol and the oxidation to E (Bellemare et al., 2005).

Furthermore, it has also been proposed that the epiandrostenediol is further converted to E by 3βHSD via its synthesis from pregnenolone in a single step through a 16-ene-synthase (Weusten et al., 1989, Starka, 2003).

The biosynthetic pathway of E is illustrated in Figure 1.16.

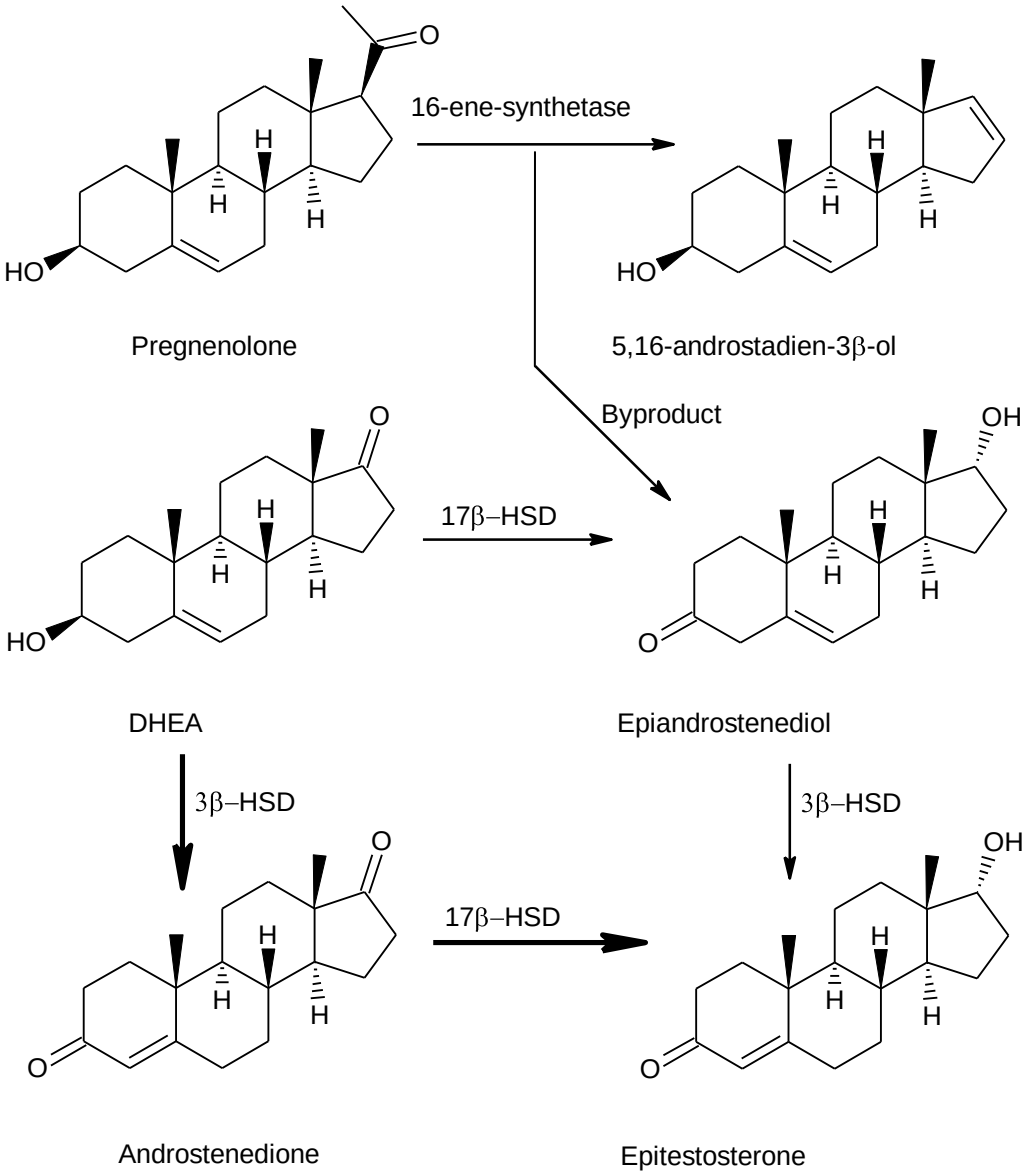


Figure 1.16 Proposed pathway for the synthesis of E. The thicker arrows represent the major routes (Weusten et al., 1989, Kicman, 2010)

1.3.4 Mechanism of action

Steroid-converting enzymes within target tissues are responsible for modulating androgen's effects at cellular level (Kicman, 2008).

Testosterone acts as a prohormone as well as a hormone, and in reproductive target tissues (cells that contain androgen receptors) and skin it may be converted by 5α-reductase into DHT, whereas T itself binds to the androgen receptor in skeletal muscle (Kicman, 2010, Greenstein,

1994c). In parts of the brain and fatty tissue, T is converted by aromatase into E_2 (Greenstein, 1994c, Kicman, 2010).

When steroid hormones bind to the androgen receptor (AR) or steroid receptor (SR) they produce a conformational change releasing a heat shock protein (HSP) from the receptor. This causes the dissociation of the receptor, already activated, from its protein and thus an increase in the DNA-binding sites hence allowing the ligand-bound receptor to bind to the hormone response element of the DNA (Kicman, 2010, Porterfield, 2001d). After dissociation, the activated androgen receptor is translocated into the nucleus and binds to the DNA thus triggering the formation of a transcription complex (Figure 1.17). The androgen receptor belongs to the steroid-thyroid-retinoic acid superfamily of receptors and comprises of a DNA-binding domain, a ligand-binding domain and an N-terminal region.

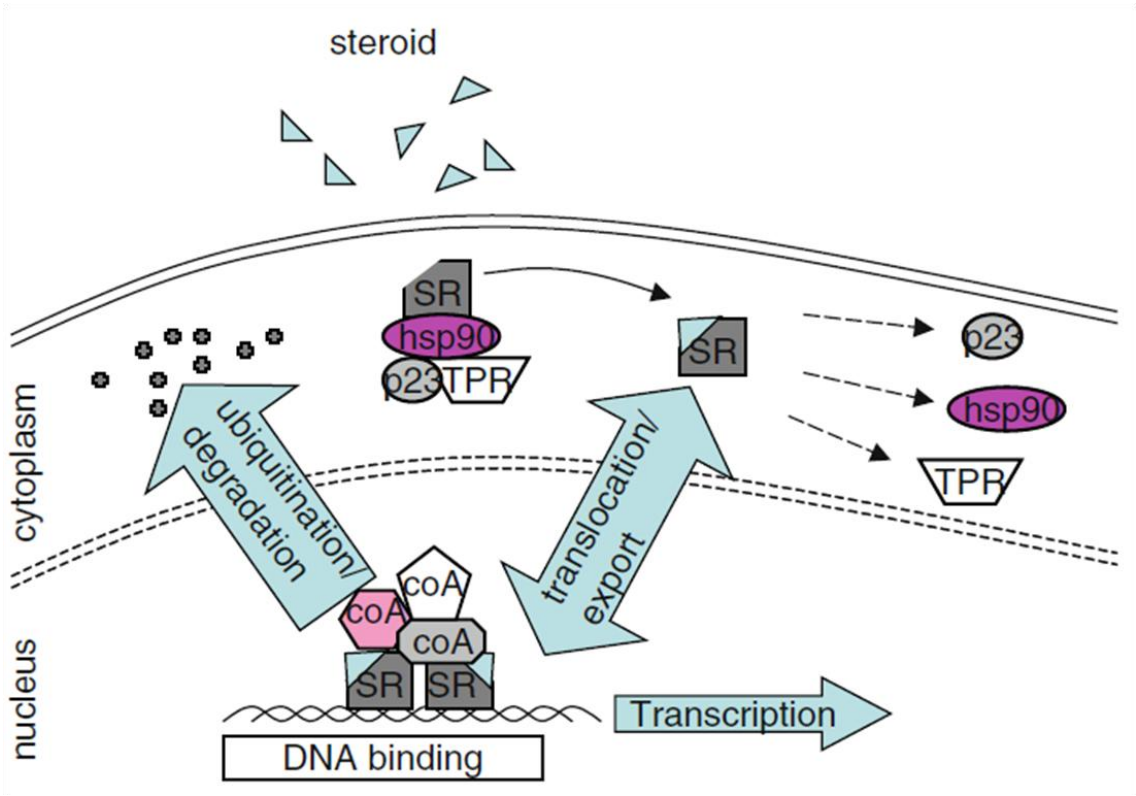


Figure 1.17 Mechanism of steroid receptor action (Kicman, 2008), based on Weigel *et al,* (Weigel and Moore, 2007a)

In the absence of hormone, the steroid receptor is an inactive oligomeric complex with the molecular heat shock protein Hsp90 and p23, and tetratricopeptide (TPR) motifs.

1.4 Pharmacokinetics of testosterone

1.4.1 Distribution, metabolism, excretion

Steroid hormones bind to plasma proteins for transport and can be found in the blood circulation as bound and unbound (Dunn et al., 1981). There are four main plasma proteins: albumin, sex-hormone binding protein (SHBG), corticosteroid binding globulin (CBG) and thyroid-binding globulin (TBG). Albumin is the principal steroid binder available in the blood stream and it will bind T, E, E_2, cortisol and P (Bruning et al., 1976, Yen and Jaffe, 1999a). The other binding globulins are available for specific hormones: SHBG is available for androgens and estrogens such as T, dihydrotestosterone (DHT) and E_2; CBG for cortisol, progesterone and T (in small amounts); and TBG for thyroxine and triiodothyronine (Yen and Jaffe, 1999b).

The clearance rate is the volume of blood completely cleared of drug and/or metabolites as measured in unit time (L/day). The clearance rate of a steroid from the total amount of blood in the body is the sum of steroid clearance rates for each tissue or organ, and the overall value will be the often referred to as metabolic clearance rate (MCR) in steroid endocrinology.

The MCR was usually measured using a continuous infusion of a radioisotopically labelled steroid at a constant rate and determining the concentration of the unconjugated radioactive steroid in peripheral venous blood. When equilibrium is reached, the concentration of the isotopic steroid in blood will be constant, the rate of clearance of the steroid from the blood will be equal to the rate of entry, and the MCR will be equal to the rate of infusion of the isotopic steroid divided by the concentration of the labelled steroid in the blood (Yen and Jaffe, 1999a). Of course, undertaking such investigations now would be met with difficulty, as ethical committees would be concerned about the administration of radio-nucleides to healthy volunteers. For this reason, stable isotopes may be administered instead and steroid concentrations measured by hyphenated mass-spectrometry.

Elimination occurs as a result of metabolic processes that occur in the kidney, liver, saliva, sweat, intestine, heart, brain or other site of the body (Centre for Cancer Education, 2007).

The main metabolites of testosterone excreted in urine are androsterone glucuronide, etiocholanolone glucuronide and, to a lesser extent, testosterone glucuronides.

In women, AD is converted to testosterone in the liver and conjugated with glucuronic acid without being released into the blood stream as free hormone (Horton and Tait, 1966). Therefore, the excretion of testosterone glucuronide in women is not a good reflection of their circulating T concentration and may not be a good way to calculate the blood concentration of testosterone (Brooks, 1984).

The MCR and the production rate of T have been determined under basal conditions (Table 1.1). The production rate is obtained by multiplying the MCR by the plasma concentration.

Table 1.1 Highly cited papers on metabolic clearance rate and production rate for T

Reference	Gender	Number of subjects	MCR (L/day)	PR (μmol/day)	PR (mg/day)
(Rivarola et al., 1966)	Male	2	1030	26.8	7.7
	Female	2	529	0.7	0.2
(Bardin and Lipsett, 1967b)	Male	6	1240	32	9.2
	Female	5	590	0.8	0.23
(Vermeulen and Ando, 1979)	Male	12	1026	26.7	7.7
	Female (postmenopausal)	10	503	0.7	0.2
(Wang et al., 2004b)	Male	11	1219	31.7	9.1
	Female	NA	NA	NA	NA

The MCR for males is about twice as high as the MCR for females, and the production rate is about 38 times higher; this might have to do with protein binding, since the MCR is inversely proportional to the concentration of SHBG in plasma, therefore women that have higher SHBG plasma concentrations than men will have a lower MCR (Mendel, 1989).

Steroids are hydrophobic substances that undergo reductive catabolic reactions through the action of enzymes to be rendered more water soluble so they can be excreted through water-soluble means (urine, sweat). Although this is not always the case, for example, the major metabolites of T, androsterone and etiocholanolone, must have very similar hydrophobicity. It is really phase 2 metabolites in the form of sulfate and glucuronide conjugates, which makes them even more water soluble and able to be excreted in urine (Gower, 1984b). Androgen metabolism and catabolic reactions happen mainly in the liver.

Testosterone is subject to phase 1 metabolism, which makes it more polar and more water soluble, oxidizing T to AD via 17β-HSD, the reduction of the A-ring to yield the 5α- and 5β-androstenediones, and via 3α- and 3β-HSD to form mainly androsterone and etiocholanone to a lesser extent (Kicman, 2010, Brooks, 1975a, Ring, 2006, Weigel and Moore, 2007b). Even after undergoing phase 1 metabolism, it is not water soluble enough to be excreted by water-mediated excreting organs. Therefore it will undergo phase 2 metabolism.

This type of metabolism excretes T as conjugated sulfate and glucuronide metabolites through the conjugation with 3'-phosphoadenosine-5'-phosphosulfate (PAPS), and uridine diphosphate glucuronic acid (UDP-glucuronic acid) respectively.

Sulfation is a high affinity, low capacity pathway because PAPS is present in the cells in low amounts. On the other hand, glucuronidation is a low affinity, high capacity pathway because glucuronic acid is present in high abundance in liver cells due to its important role in glycolysis and glycogenesis. When high doses of androgens are administered, due to the low availability of PAPS the remainder will undergo glucuronidation, even though the initial preferred pathway is sulfation.

Metabolic pathways of T can be seen in Figure 1.18 and, although testosterone can be excreted as a sulfate conjugate, only the glucuronide is shown as it is excreted in higher amounts (Gower, 1984b).

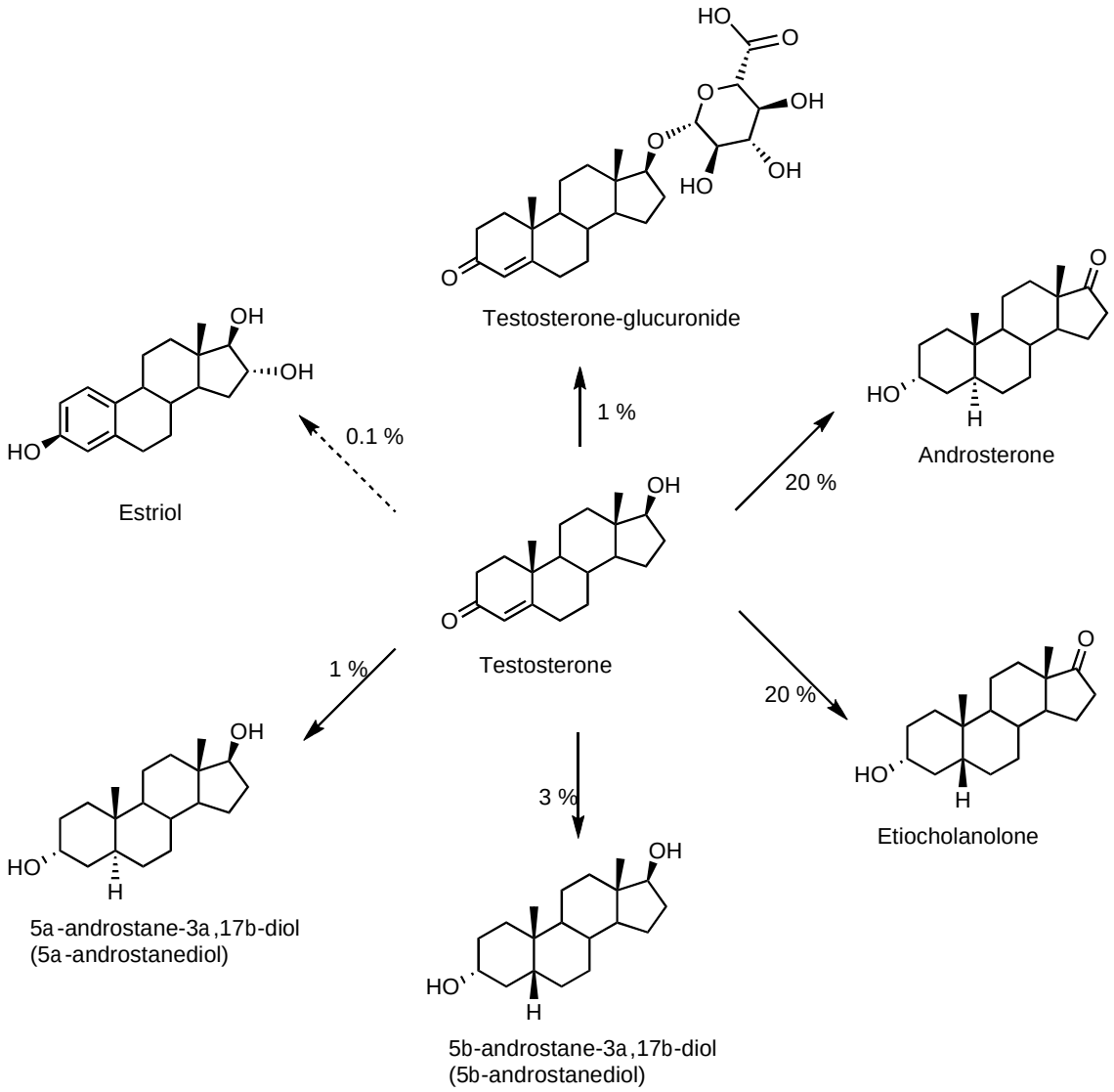


Figure 1.18 Metabolic pathway of T (Brooks, 1975a)

1.4.1.1 Glucuronidation pathways

Steroid glucuronic conjugates are excreted in urine when glucuronic acid is transferred from UDP-glucuronic acid to the aglycone moiety catalysed by glucuronosyltransferase enzymes (UGTs). The UDP-UGT gene encodes enzymes that will link glycosyl groups covalently

(glucose, glucuronic acid, xylose and galactose) to lipophilic substrates to render them more hydrophilic (Guillemette, 2003).

The addition of a sugar acid to a steroid increases its hydrophilicity, helping with its urinary excretion.

The mammalian UGT gene has 117 members divided into four families: UGT1, UGT2, UGT3 and UGT8, with UGT2 divided into 2 subfamilies: UGT2A and UGT2B (Mackenzie et al., 1997, Mackenzie et al., 2005). The enzymes from UGT1 and UGT2 donate more readily the glucuronic acid, but UDP glycose and UDP xylose are also available. The UGT3A1 uses UDP N-acetylglucosamine; UGT3A2 uses UDP glucose and UDP xylose, but not UDP N-acetylglucosamine (MacKenzie et al., 2011). The sugar donor that UGT8 uses is UDP galactose (Ichikawa et al., 1996).

There are twenty-seven active human enzymes (Mackenzie et al., 2005) but only four have significant activity concerning androgen metabolism: UGT2B4, UGT2B7, UGT2B15 and UGT2B17 (Sten et al., 2009b). UGT2B4 is the one with lower steroid glucuronidation activity.

UGT2B7 is specific for a wide range of steroids, including C19 steroids with a hydroxyl function at the 3α-, 16α-, and/or 17α positions. Its glucuronidation activity includes 5α-androstan-3α-17β-diol (5α-diol) and E (Girard et al., 2003, Sten et al., 2009a, Sten et al., 2009b, Coffman et al., 2003).

UGT2B15 catalyzes the major androstanediols (5α-and 5β-androstane-3α,17β-diol) , but it shows a higher stereoselectivity towards the first one (Sten et al., 2009b). This enzyme specifically conjugates 17-hydroxyl groups.

UGT2B17 has a high capacity for 5α-DHT conjugation, and mainly testosterone glucuronidation (Beaulieu et al., 1996, Sten et al., 2009a). Although it does not glucuronidate E, it binds it with a similar affinity as T.

UGT2B7 and UGT2B17 exhibits the highest activity rates in the glucuronidation of androsterone and etiocholanolone, both being excretion products of T (Sten et al., 2009b).

In doping in sports the method to test for testosterone abuse is through a urinary T/E ratio obtained via GC-MS, as mentioned previously. Testosterone is mainly excreted as a glucuronide conjugate which is subjected to a hydrolysis step to cleave the glucuronide and thus only the steroid moiety undergoes the remaining analysis. There is, therefore a high interest in testosterone glucuronidation. The large variation in testosterone glucuronide (TG) excretion is associated with a genetic polymorphism in UGT2B17 (Schulze et al., 2008, Jakobsson et al., 2006, Sten et al., 2009a).

If an individual carries a *del/del* genetic polymorphism in the UGT2B17 gene, the excretion of TG will be approximately 20 times inferior to those of individuals with an *ins/ins*. (Schulze et al., 2008).

This will ultimately affect the T/E ratio for doping purposes, with an athlete carrying the *del/del* polymorphism being able to mask any exogenous T administration.

1.4.1.2 Sulfation pathways

Sulfoconjugation of steroids involves the transfer of a sulfonate group (SO_3) from PAPS to an hydroxyl group, via a sulfotransferase enzyme (Strott, 1996) to form a sulfate (SO_4). Sulfation occurs mainly in the liver, but it can also occur in the testes and adrenal gland (Jaffe and Payne, 1971, Ruokonen et al., 1972, Falany et al., 1989).

There are two types of sulfotransferase enzymes: cytosolic and membrane associated, but only the cytosolic ones are involved in steroid metabolism.

There are ten human sulfotransferase genes encoding eleven cytosolic sulfotransferase proteins (Glatt and Meinl, 2004). The SULT2 family is the most relevant for hydroxysteroids, with SULT21A being more active for DHEA and able to conjugate androsterone and T as well.

The clearance rate of steroid sulfates is low when compared to unconjugated steroids, mainly because of their affinity to albumin, thus showing that they are not directly excreted from the body (Strott, 1996). For example, the average clearance rate of DHEA in men is 1059 L/day and 854 L/day in women; the corresponding sulfate having a clearance rate of 16.7 L/day in men and 16.5 L/day in women (Wang et al., 1967). With T, the average clearance rate in men is

225 L/day and in women 2029 L/day. The sulfate is cleared in men at an average rate of 24.5 L/day and in women at 21.5 L/day (Wang et al., 1967).

Generally, it is thought that steroid sulfates form a pool of inactive steroids in the blood circulation which can be locally activated by sulfatases.

Due to the low excretion of testosterone sulfate, this metabolite is not a good biomarker for testosterone abuse (Schulze et al., 2011).

1.5 Plasma binding proteins & testosterone

Steroid hormones bind to plasma proteins for transport and can be found in the blood stream as bound and unbound (Dunn et al., 1981). There are four binding proteins in blood: albumin, sex-hormone binding globulin (SHBG), corticosteroid binding globulin (CBG) and thyroid-binding globulin (TBG). Out of these four only the first three have the ability to bind testosterone to any extent (Dunn et al., 1981, Bruning et al., 1976, Yen and Jaffe, 1999a, Yen and Jaffe, 1999b).

The affinity of an analyte to a protein is the tendency it has to be bound to that protein. The higher the affinity between them, the more tightly bound the analyte is. Testosterone's affinity with albumin is low (K_a~ 3.6 x 10^4 M^{-1}) when compared to SHBG (K_a= 1.6 x 10^9 M^{-1}) (Table 1.2).

Although testosterone's affinity is lower with albumin, it is of high capacity since albumin accounts for ~60 % of proteins in plasma. The binding capacity is a measure of the quantity of analyte that can be bound by a given protein.

The higher binding affinity of T with SHBG is due to its high affinity for 17β-hydroxy-androgens and estradiol (Wheeler, 1995, Dunn et al., 1981).

Testosterone binds mainly to SHBG followed by albumin and CBG although to lower extent with the latter (Wheeler, 1995). It is estimated that, of the circulating T in adult men, approximately 45% is bound to SHBG, 50% to albumin and the other 5% circulates as free and bound to CBG (Karagiannis and Harsoulis, 2005).

For an estimate of the bound circulating T in eugonadal males and females, please see

Table 1.3 and Figure 1.19 (Dunn et al., 1981). For the concentrations in mass see Table 1.4.

Steroid binding is temperature and pH dependant, and it is 2.5 times higher at 4 °C than 37 °C. It becomes irreversibly destroyed at temperatures higher than 60 °C and at pH below 5 (Selby, 1990).

Alterations in binding may lead to changes in peripheral metabolism and alter T concentration in serum (Gordon et al., 1976b).

Table 1.2 Binding proteins' association constants for testosterone in blood

Binding protein	Binds to	Association constant (M^{-1})	MW of binding protein (kDa)	Association constant ($g.L^{-1}$)
Albumin	Testosterone, estradiol, cortisol & progesterone	3.6×10^{4}	60	9.6×10^{-8}
SHBG	Testosterone, dihydrotestosterone & estradiol	1.6×10^{9}	115	2.2×10^{-12}
CBG	Testosterone, cortisol & progesterone	5.3×10^{6}	50	6.6×10^{-10}

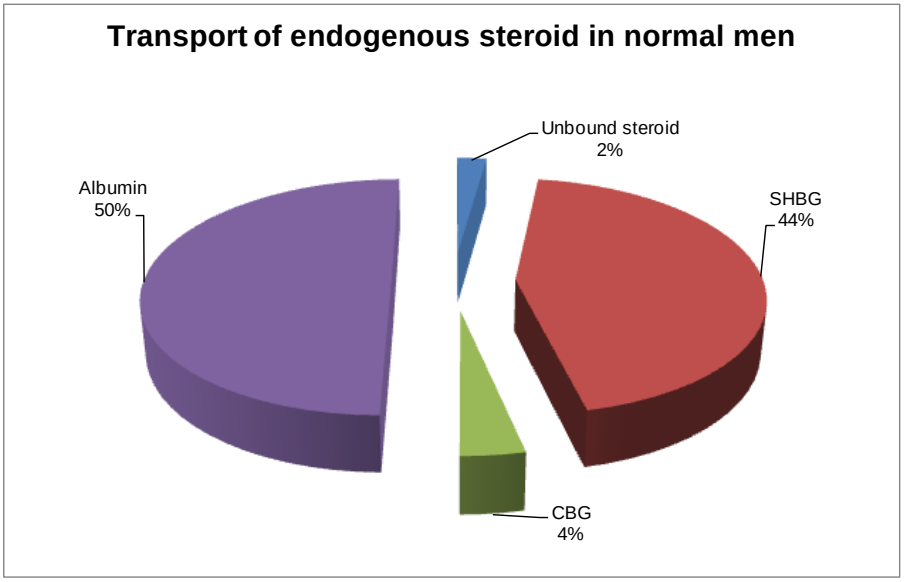
Table 1.3 Transport of endogenous T (Dunn et al., 1981, Nisula et al., 1978)

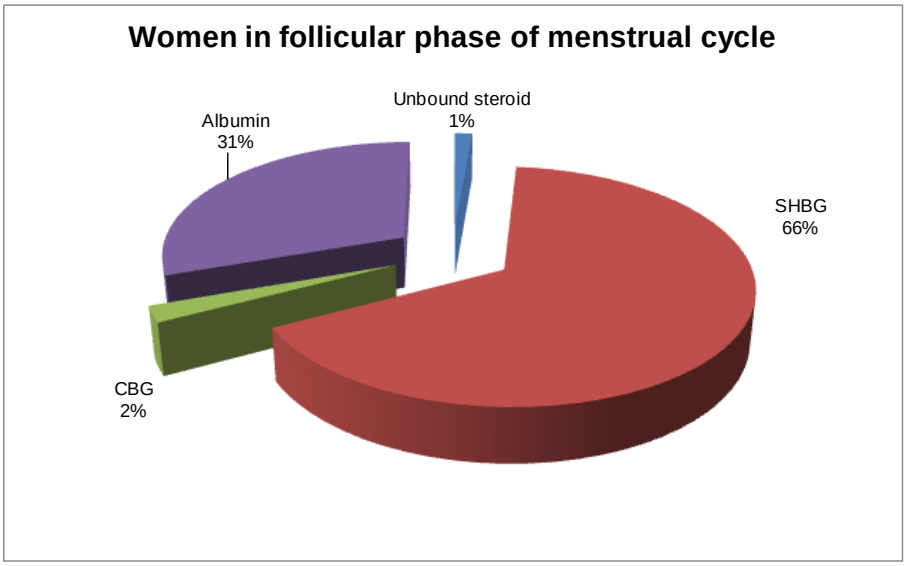
Gender	Total T (nM)	Total SHBG (nM)	Total albumin (mM)	Total CBG (μM)	T bound to SHBG (%)	T bound to albumin (%)	T bound to CBG (%)	Unbound T (%)
EM	23	28	0.56	0.7	44.3	49.9	3.56	2.23
EF-FP & LP[2]	1.3	37	0.56	0.7	66	30.4	2.26	1.36

Table 1.4 Transport of endogenous T

Gender	Total T (nM)	Total T (ng/mL)	T bound to SHBG (ng/mL)	T bound to albumin (ng/mL)	T bound to CBG (ng/mL)	Unbound T (ng/mL)
EM	23	6.6	2.9	3.3	0.2	0.1
EF-FP & LP	1.3	0.4	0.2	0.1	0.01	0.01

[2] EM stands for eugonadal men and EF for eugonadal females. LP stands for lutheal phase and FP for follicular phase





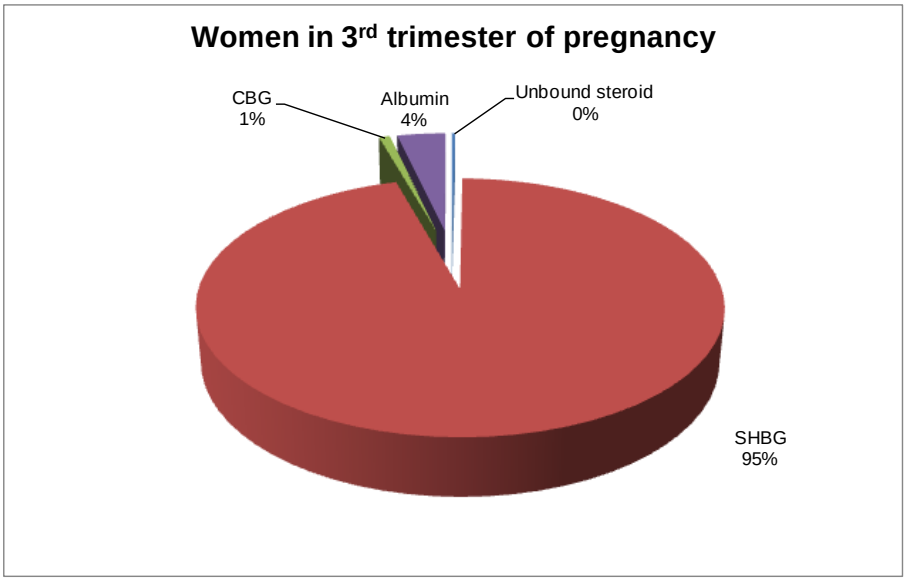


Figure 1.19 Proportion of T bound to plasma proteins, based on data by Dunn *et al.* (Dunn et al., 1981)

SHBG is a homodimeric glycoprotein synthesized in the liver and is made by the association of two identical subunits differently glycosylated (Yen and Jaffe, 1999b). Each monomer has 373 amino acid residues that are released along with water from a condensation reaction between amino acids, and a tandem series of laminin G-like domains (Figure 1.20). (Walsh et al., 1986). Laminins are large heterotrimeric glycoproteins that play a key role in membrane architecture and function (Tisi et al., 2000).

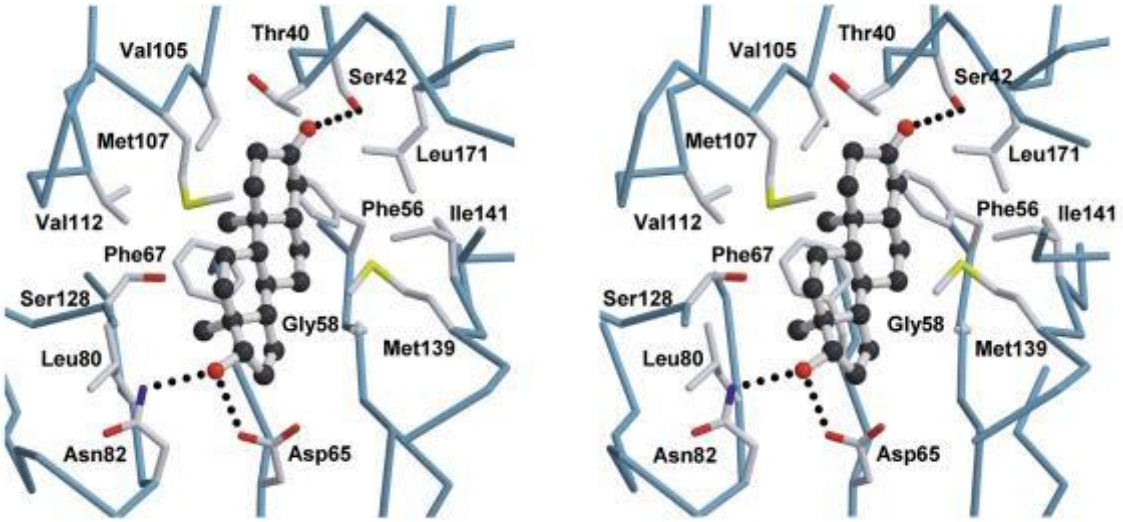


Figure 1.20 - Representation of two homodimeric SHBG showing its two steroid-binding sites. The steroid present is 5α-DHT (Grishkovskaya et al., 2000)

It used to be thought that there was one binding site per mole leading to the assumption that a single steroid-binding site could be found in the dimer interface (Hammond, 1990, Petra, 1991). However, recent crystal studies (Avvakumov et al., 2001) have shown that each monomer in the homodimeric SHBG protein has one binding site (Grishkovskaya et al., 2000) amounting to two binding sites per protein.

1.5.1 Hormone availability

Regarding hormone availability for tissues there are two different theories: the free hormone and the bound hormone.

Proponents of the free hormone fraction theory argue that this form is the main component of most hormone biological activity, and only in this fraction will the hormone be able to reach target tissues (Mendel, 1989, Ekins, 1990, Brooks, 1984) and, in the absence of significant cellular metabolism, the concentration of free hormone in nuclear compartments is the same as measured *in vitro* (Pardridge, 1988). This idea mainly comes from observing abnormal concentrations of protein bound hormone and its hormonal effects correlating with the free hormone concentration rather than that of the bound (Ekins, 1990).

In vitro studies have been performed to assess the amount of free testosterone available to tissues in the body.

It seems unlikely that only the free T reaches certain tissues, especially the liver due to its high concentration in this organ. The intracellular concentration of testosterone present in the liver is thought to come from the rapid dissociation of testosterone from albumin during sinusoidal transit and there may be little or no contribution from SHBG bound T due to its much higher affinity constant (Mendel, 1989). This is sustained by the second theory that claims plasma steroid-binding proteins are found in target tissues because the protein-bound fraction is also available for them and that the rate limiting effects of protein-bound hormone dissociation may affect hormone uptake, thus invalidating the first hypothesis (Siiteri et al., 1982, Pardridge, 1981). Studies on the albumin-bound fraction indicate that this bound-protein has an influence in the free steroid theory due to the rapid equilibrium between binding and dissociation (van den Beld et al., 2000, Mendel, 1990). Plasma binding proteins have not been proved solely to help steroid entry into cells, but this has not been disproved either.

In summary, the free hormone hypothesis is currently the most accepted, but it is not flawless as it does not explain the availability of every hormone and the existence of specific binding proteins in certain target tissues is yet to be determined (Mendel, 1989). The concentrations of hormones are measured in serum bearing in mind that only free hormones are physiologically active and that their *in vitro* concentrations are indicative of an *in vivo* behaviour even though the relationship between bound and free hormone is a dynamic one (Ekins, 1992). Therefore, changes in the blood flow, composition or temperature can change the free hormone level as studied *in vitro* (Pardridge, 1988, Yen and Jaffe, 1999b).

Whether the reason for the of remaining existence of these two theories rather than just one after so many years is due to the difficulty carrying out this type of research (will the experiments performed *in vitro* ascertain that the same conditions happen *in vivo* in terms of hormone availability to all the organs in the body), or the importance this has to the scientific community is a matter of speculation.

The plasma concentration of T is 20 times higher in males than in females reaching concentrations of 26 nM (7 ng/mL) for males and 1.3 nM (0.4 ng/mL) for females, as calculated from the data associated with the selected studies from Table 1.5.

Table 1.5 Testosterone's concentration in serum

Reference	Subjects	Number of samples	Concentration in plasma (nM)	Concentration in plasma (ng/mL)
(Bardin and Lipsett, 1967a)	Normal men	24	26	7.5
	Normal women	20	1.4	0.4
(Gandy, 1977)	Normal men	NA	23	6.6
	Women in LP & FP	NA	1.3	0.4
(Gaidano et al., 1980)	Normal men	20	26	7.5
(Dunn et al., 1981)	Normal men	NA	23	6.6
	Women in LP & FP	NA	1.3	0.4

1.6 Analysis of testosterone & epitestosterone – misuse in sports

Analytical screening and confirmation method for anabolic steroids is performed using gas-chromatography mass-spectrometry (GC-MS) (Donike et al., 1983, Wheeler, 1993, Weathermon and Crabb, 1999). The ratio of the aglycone and the unconjugated urinary T and E is approximetely 1 (Donike et al., 1983, Van Renterghem et al., 2010). This screen also incorporates a test for testosterone administration, based on the determination of the ratio of testosterone to epitestosterone (aglycone plus free steroid). If the T/E ratio exceeds a threshold of 4, then further confirmatory tests need to be performed (Figure 1.21) (World Anti-Doping Agency, 2004).

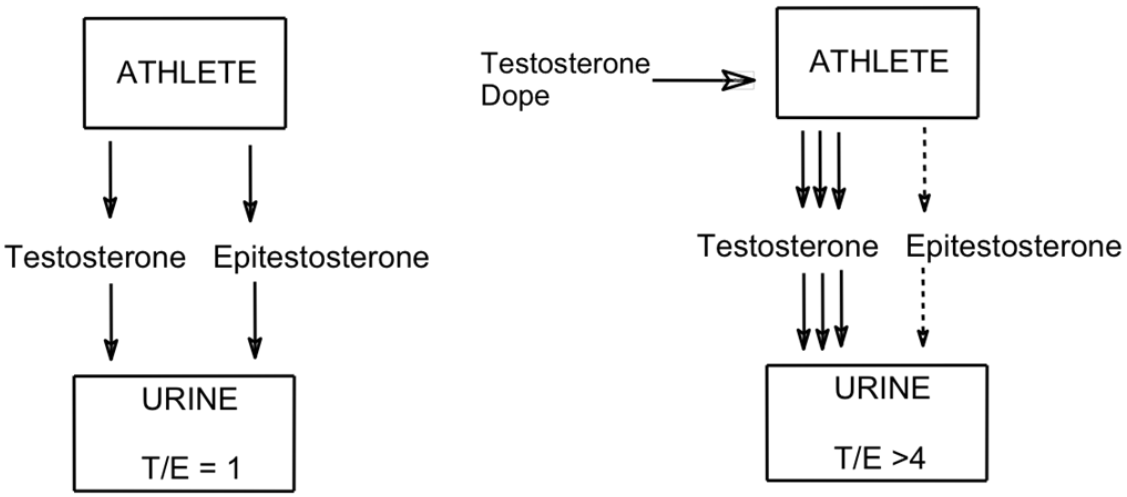


Figure 1.21 Diagram showing normal T/E and above the threshold T/E

About 1 % of T and 30 % of E is excreted in urine unchanged (Brooks, 1975a, Wilson and Lipsett, 1966), and intramuscular injection of both at a ratio of 30:1 might give a T/E of approximately 1 (Kicman et al., 1990). However, because the increase in T concentration would provoke a decrease in LH, a urinary T/LH immunoassay test for males is used as a marker of T intake, since the ratio would substantially decrease (Kicman et al., 1990). There have also been reported cases of athletes with a naturally occurring T/E > 4 (Carlstrom et al., 1992, Dehennin and Matsumoto, 1993, Kicman et al., 1993, Catlin et al., 1997, Kicman and Gower, 2003), and the use of this ratio as a marker of T intake also helps to diferentiate false positives. Further confirmatory tests using gas-chromatography carbon ion-ratio mass-spectrometry (GC-C-IRMS) are also performed.

More recently, the introductin of the athlete's biological passport may help monitor T/E levels, as well as other performing enhancing drugs (Sottas et al., 2010). This profiling method uses the athlete's individual values for within-subject comparison purposes, rather than a population based value. Such monitoring method is particularly useful when the marker presents a low ratio of within-subject to between-subject variations.

Testosterone has a molecular weight of approximately 288 $g.mol^{-1}$. During derivatisation, two trimethylsilyl (TMS) (molecular weight of 72 $g.mol^{-1}$) groups are attached to T via the hydroxyl groups. The resulting mass of the testosterone di-TMS is thus 432 $g.mol^{-1}$.

An internal standard is also added to each urine sample to account for any product losses during extraction or analysis. The internal standards used are trideutero testosterone, with deuterium (atomic mass = 2) replacing hydrogen (atomic mass = 1) at positions C16 (twice) and C17. Thus, the molecular weight of the deuterated derivatised testosterone is 435 $g.mol^{-1}$.

Epitestosterone, being the 17-epimer of testosterone, has the same molecular weight but the two can be distinguished chromatographically.

1.7 Alcohol & hormones

1.7.1 Alcohol consumption

Ethanol (EtOH) is most commonly known as alcohol, a sociably accepted drink that despite having its benefits (boost of cardiovascular health) also causes addiction (Sadler, 2007). The effects alcohol can cause in the human body tissues are dependent upon the blood alcohol concentration (BAC), which is determined by alcohol's absorption rate, distribution, metabolism and excretion; and affected by the rate consumed, presence of food in the stomach, type of alcoholic beverage and genetic factors such as variations in the main metabolic enzymes (Zakhari, 2006, Jones, 2011).

The metabolism of alcohol results in the production of acetaldehyde, a toxic byproduct which is responsible for tissue damage, formation of damaging molecules known as reactive oxygen species (ROS) and a change in the redox state of cells present in the liver (Zakhari, 2006).

One unit of alcohol is the equivalent to one standard drink which is approximately 8 grams (g) of alcohol.

The recommended maximum alcohol intake for males and females is shown in Table 1.6.

Table 1.6 Alcohol consumption in males and females adapted from Sadler *et al.* (Sadler, 2007)

Alcohol consumption	Males (g/ week)	Females (g/ week)
Safe	168 – 224 (24 – 32 g/day)	112 – 168 (16 – 24 g/day)
Hazardous	168 – 400	112 – 280
Dangerous	> 400	> 240

For convenience it is normal to write alcohol consumption in terms of units. One unit of alcohol is the equivalent to one standard drink, which is approximately 8 grams of alcohol.

Moderate consumption of alcohol is seen as socially acceptable and a male or female is considered a social drinker if they drink an alcohol beverage occasionally or regularly in moderation. A heavy drinker is someone that drinks heavily and regularly and normally above 7 units/day in males, and above 5 units/day in females. A binge drinker drinks irregularly and heavily.

The alcohol content of a drink is expressed in terms of percentage. For example, a pint of beer with 4.5 % alcohol by volume means that there is 4.5 mL of alcohol in 100 mL of beer.

1.7.2 Absorption, distribution, metabolism & excretion

Of all the alcohol ingested, 20 % is absorbed in the stomach and 80% in the upper small intestine by passive diffusion (diffusion across a membrane) providing the alcohol concentration in the stomach and small intestine exceeds that in blood (Sadler, 2007).

The BAC rises according to how fast the alcohol leaves the stomach and the extent of this first pass metabolism (through stomach and liver) where alcohol's concentration is reduced before it reaches the systemic circulation (Zakhari, 2006).

Absorption is normally complete in 1 – 3 h and is normally faster if the individual has an empty stomach since the alcohol will pass more rapidly to the small intestine. This happens with diluted mixtures of alcohol, not in the case of spirits.

Alcohol is metabolised into an aldehyde via alcohol dehydrogenase (ADH) mainly present in the liver and some in the stomach; and by CYP450 enzymes and catalase in non-liver tissues such as the brain. This metabolism may be oxidative (add oxygen or remove hydrogen), or nonoxidative.

Oxidative alcohol's metabolism via ADH is the main metabolic pathway of alcohol in the liver. This results in the production of acetaldehyde, which is a toxic byproduct responsible for tissue damage and possibly the addictive process (Figure 1.22) (Zakhari, 2006).

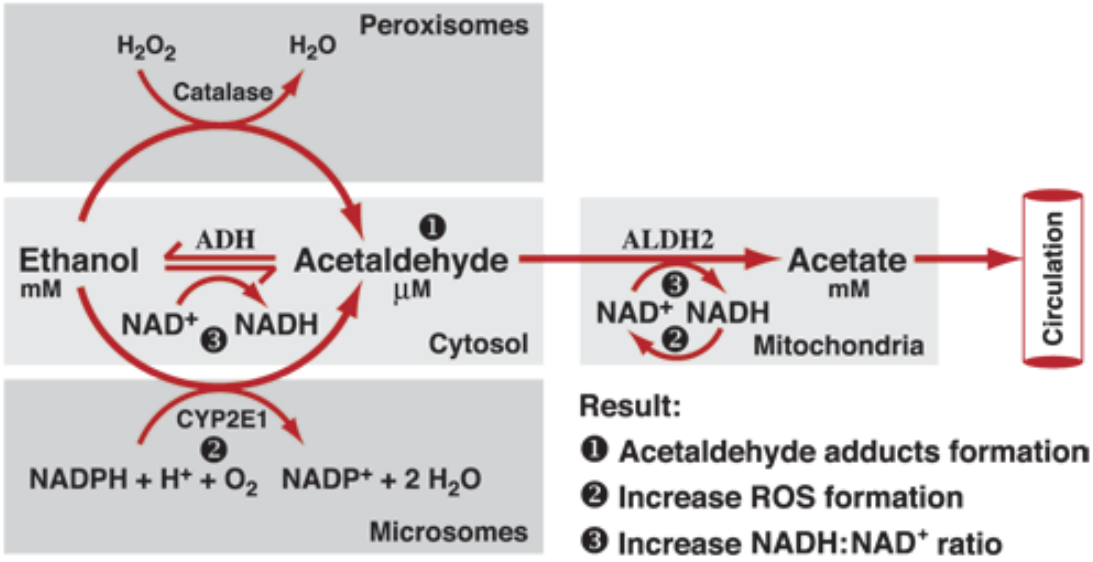


Figure 1.22 Metabolism of alcohol (oxidative pathway) (Zakhari, 2006)

ADH is found in the cytosol (cell's fluid) and there are five types in humans. ADH1 and ADH2 are found in higher quantities in the liver and are mainly responsible for alcohol's metabolism (Ring, 2006).

When at high concentrations, alcohol is eliminated more rapidly due to the presence of enzymes with high activity levels such as ADH4 and ADH1B. This oxidative process involves the reduction of NAD^+ by two electrons to NADH, which acts as an intermediate carrier of electrons. This whole oxidative process results in hepatocytes (liver cells) being more prone to

the consequences of alcohol's byproducts (free radicals and acetaldehyde) resulting in liver damage (Zakhari, 2006).

Cytochrome's P450 isoenzymes CYP2E1, 1A2 and 3A4, all of which are present in microsomes, are also involved in oxidative alcohol's metabolism in the liver. The first enzyme, CYP2E1, is present not only in the liver but also in the brain where ADH activity is smaller. It is stimulated by chronic alcohol consumption and acts when alcohol is present at high concentrations metabolizing it into acetaldehyde.

Catalase, found in peroximes[3], also oxidises ethanol *in vitro* in the presence of NADPH, a H_2O_2 generating system but this is considered a minor pathway in alcohol's oxidation. Nevertheless, catalase may have an important role in alcohol's metabolism in the brain that can lead to alcohol addiction, as discussed elsewhere (Zimatkin and Deitrich, 1997).

Regardless of the path ethanol undertakes to be oxidised into acetaldehyde, there are two main products as a result of this process: acetaldehyde and acetate.

Acetaldehyde is highly toxic, hence why few studies directly involving acetaldehyde have been performed in humans. Studies in humans have been mainly performed involving carriers of the deficient gene variant (allele) that encodes acetaldehyde dehydrogenase (ALDH), ALDH2*2, which produces an inactive ALDH enzyme. Carriers of this allele (40% of East Asian population (Ring, 2006)) cannot metabolise acetaldehyde, which accumulates in the body and increases peripheral concentration. If the concentration of acetaldehyde increases by inhibition of ALDH, it is then very difficult to determine its dose-response pattern (Quertemont and Didone, 2006).

Some of acetaldehyde's physiological effects include increased skin temperature and facial flushing, increased heart and respiration rates, lowered blood pressure, narrowing of the airways, nausea and headache. For the mechanisms that induce these symptoms the reader is referred to the article by Quartermont *et al.*(Quertemont and Didone, 2006).

Acetaldehyde is oxidized in the liver by ALDH to form acetate, which then enters the peripheral circulation. It is oxidised into carbon dioxide (CO_2) in the heart, skeletal muscle and brain cells.

[3] Peroximes are an organelle found in eukaryotic cells that contain enzymes like catalase and urate oxidase. They are surrounded by only a single membrane, and they do not contain DNA and exist with a genome of their own.

It is then metabolised into acetyl coenzyme-A, which in turn is involved in lipid and cholesterol biosynthesis (Zakhari, 2006). Physiologically, acetate increases blood flow into the liver and depresses[4] the central nervous system (CNS).

Alcohol may also be metabolised via a non-oxidative pathway. Although this pathway is minimal, it will be briefly mentioned.

Through this pathway, alcohol may be metabolised in two different ways. One will lead to the formation of fatty acid ethyl esters (FAEE), involving the reaction of alcohol with fatty acids; whereas the other one leads to the formation of phosphatidyl[5] ethanol (Zakhari, 2006).

Chronic alcohol consumption causes an initial increase in estrogens in postmenopausal females, followed by a decrease (Gavaler and Vanthiel, 1992). Moderate consumption, in females, induces a rise in estrogen possibly due to the increased rate of aromatisation of testosterone to estradiol (Gavaler et al., 1993). This rise may reduce the risk of cardiovascular disease, but one must not forget the risk of breast cancer associated with it. In males, chronic alcohol consumption causes a decrease in T, which will in turn lead to testes atrophy, impotence and loss of secondary sexual characteristics (Emanuele and Emanuele, 2001).

Elimination of alcohol follows a zero-order kinetics in which ethanol is being eliminated at a constant rate from unit of time, when ingested in lower amounts (blood alcohol concentration of up to 0.2 g/L). Excretion is a first-order process, hence more alcohol is excreted unchanged with higher amounts (Jones, 2011).

Excretion of alcohol is done in breath, urine and sweat (3 – 10 %), and as its conjugates ethyl glucuronide (EtG) and ethyl sulphate (EtS) (< 1 %) (Jones, 2011).

The rate of elimination of ethanol varies accordingly to gender, diet, age, time of day and degree of alcohol consumption.

The average blood alcohol clearance rate by the liver in a healthy person is 15 mg of alcohol/100 mL of blood/h (equivalent to 1 unit/h), with the range being 10-40 mg/100 mL of blood/h (Sadler, 2007).

[4] Depression of the CNS involves decreased rate of breathing, decreased heart rate, and loss of consciousness possibly leading to coma or death.
[5] A type of lipid molecule containing phosphorous.

A typical blood alcohol concentration curve depends on absorption, metabolism and excretion. It starts off with the alcohol concentration reaching a maximum, then a fall due to diffusion in the tissues to reach an equilibrium (which takes approximately 15-30 min), falling progressively in a linear way (elimination) (Sadler, 2007).

At high blood alcohol concentrations such as 200 mg %, the decrease is not linear due to losses via breath and urine. In cases such as these it takes about 12 h to eliminate this amount.

In summary, approximately 3 – 10 % of alcohol is excreted in breath, urine and sweat, less than 1 % as conjugates.

1.7.3 Alcohol & the endocrine system

1.7.3.1 HPA axis & alcohol

Cushing syndrome is a disease arising from excess cortisol, which is sometimes caused by an excess of prescribed glucocorticoids, or from ACTH-producing pituitary tumours.

This disease resembles that of alcohol's effects in humans. Symptoms of this disease include redistribution of fat causing central obesity and purplish stretch marks, round red face ('moon' face) and osteoporosis. In women, terminal hair on the face may appear and also menstrual irregularities (Emanuele and Emanuele, 1997). Alcohol drinkers develop a pseudo-Cushing syndrome physically indistinguishable from the true one, although it is milder. It is called 'pseudo' because its effects disappear after 2-4 months of alcohol abstinence.

The similarities are also noticeable on a biochemical level with glucose intolerance, high plasma cortisol concentrations, loss of normal diurnal variation of cortisol in plasma and increased urinary glucocorticoid excretion (Cobb and Vanthiel, 1982).

It is still unclear whether this disease originates from alcohol's effects on the brain, pituitary or adrenal glands but it is an indication of alcohol disturbing the HPA axis.

Studies in chronically intoxicated mice showed a decrease in corticosteroid plasma level after an acute alcohol ingestion (1.6 g/kg body weight) when compared to control mice kept on a water based intake (Kakihana et al., 1971). The rate of corticosterone metabolism in chronically

intoxicated mice was no different from the controls, hence the differences between the two groups could not be justified as an influence of alcohol in corticosterone metabolism.

Further studies on mice's ACTH pituitary levels showed no increase after the same alcohol dose (1.6 g/kg body weight). This suggested that alcohol may not have been stimulating the adrenal glands by releasing ACTH, which contradicts previous studies that supported the hypothesis that the pituitary gland was stimulated before the adrenal glands (Noble et al., 1971). This was also supported with evidence from another study, in which rats were administered alcohol and their ACTH levels had not increased when compared to the control group (Gambert et al., 1981).

However, later studies in the 80s showed that acute alcohol administration (3.2 g/kg body weight of 12% ethanol solution in physiological saline) had the ability to activate the HPA in animals by significantly increasing plasma levels of ACTH, corticosterone and plasma catecholamines (Thiagarajan et al., 1989). These findings were in accordance with previous studies in which a single alcohol dose that raised blood ethanol concentration to more than 100 mg/100 mL resulted in increased plasma concentration of ACTH and glucocorticoids (Noth and Walter, 1984, Guaza et al., 1983, Rivier et al., 1984, Rivier and Lee, 1996).

In rats with chronic alcohol consumption, the HPA function decreases and therefore plasma ACTH and corticosterone levels decrease (Nolan et al., 1991).

These discrepancies, although not entirely clear, may be due to dose response problems.

In humans, studies with a control group and alcoholic patients where they were administered 1 mL EtOH/kg of body weight (19.7 g of alcohol) showed an increase in urinary and plasma 17-hydroxy corticoids, while 17-ketosteroids remained the same (Kissin et al., 1960).

Another study done in the 60s (Mendelson and Stein, 1966) showed that the effect of alcohol in non-alcoholic volunteers was an increase in cortisol serum concentration only when the volunteer developed gastrointestinal distress due to drinking. In comparison, alcoholic drinkers had high cortisol serum concentration in response to alcohol administration even when drinking was not linked to alcohol-induced gastrointestinal distress. However, these volunteers had their

higher cortisol serum levels when they had stopped drinking, suggesting that the cortisol increase was stimulated by stress rather than an adrenal direct stimulation.

These studies were supported with evidence showing that acute alcohol administration in volunteers and in chronic alcoholics resulted in an increase in hydrocortisone concentration when the blood alcohol concentration was higher than 100 mg % (Fazekas, 1966, Jenkins and Connolly, 1968, Bellet et al., 1970, Stokes, 1973, Merry and Marks, 1972).

A recent study in volunteers with acute alcohol intoxication (AAI) also showed ACTH and cortisol significantly increased and that the increase was higher in women than in men (Frias et al., 2002).

1.7.3.2 HPG axis & alcohol

Chronic alcohol consumption has long been associated with the increase in 5α-reductase activity in the liver, a rate limiting enzyme for the metabolism of T in the liver thus enhancing the catabolism of T (Rubin et al., 1976).

In alcoholics, FSH, LH, and E_2 levels were significantly increased, and T and progesterone levels were significantly decreased (Muthusami and Chinnaswamy, 2005). No significant change was noted in prolactin levels. Semen volume, sperm count, motility, and number of morphologically normal sperm were significantly decreased. Chronic effect appears to have an effect on the hypothalamic-pituitary-gonadal axis by inhibiting testicular steroidogenesis, as well as by blocking the release of LH and its releasing hormone.

Acute intoxication in men can cause a temporary decrease in serum T (Frias et al., 2000, Frias et al., 2002, Karila et al., 1996). Since T decreases, LH values were expected to increase due to the negative feedback mechanism. However, this did not occur, which shows a defect in the human hypothalamic-pituitary-gonadal unit (Cicero, 1982).

Acute alcohol intoxication in healthy premenopausal women (0.4 – 0.5 g/kg) shows a rise in plasma T, a decline in AD, an increase in the T/AD urinary ratio and a decrease in urinary androsterone and etiocholanolone (T's main catabolic metabolites) (Sarkola et al., 2001). Other

studies corroborate this (Karila et al., 1996, Frias et al., 2000). Regarding urinary T/E, acute alcohol intoxication significantly increases this ratio (Falk et al., 1988, Karila et al., 1996, Seppenwoolde-Waasdorp et al., 1996, Geyer et al., 1996, Mareck-Engelke et al., 1996).

More details of acute alcohol intoxication and testosterone may be found in the following section.

1.8 Why are we interested in the influences of alcohol on T?

The effects of acute alcohol intoxication on the endocrinology, metabolism and excretion of T in the male is being investigated as the result of several reported findings of T/E increase after alcohol intake (Falk et al., 1988, Karila et al., 1996, Seppenwoolde-Waasdorp et al., 1996, Geyer et al., 1996, Mareck-Engelke et al., 1996), the most recent one being a sports tribunal where the defendant argued that his high urinary T/E ratio was caused by the fact that he had been drinking the night before. A summary of these results may be found in Table 1.7 at the end of this section, after a more detailed analysis on the published data.

In 1988, an anti-doping laboratory in Sweden found a positive result for testosterone, and the athlete justified it with previous alcohol ingestion. A study was performed by giving two different doses of alcohol to 4 healthy male volunteers over a 4 h period in the morning (Falk et al., 1988). The first dose, acting as a pilot study, at 0.8 g per kilogram body weight (g/kg) had a negligible effect on the T/E ratio. The second dose at 2 g/kg showed an increase in T/E ratio in urine from 1.14 to 1.52 within 14 h, with one subject showing an increase of 90%. Samples were collected at 0 h, 6 h, 14 h and 22 h. The authors speculated that the "increased $NADH/NAD^+$ ratio obtained in connection with the metabolism of ethanol may reflect a block in oxido-reductive formation of androstenedione from endogenous testosterone. Because the increased $NADH/NAD^+$ ratio is likely to displace the equilibrium between testosterone and androstenedione towards testosterone, the concentration of testosterone may increase".

In 1996, more studies were performed by different research groups.

One analysed serum T concentration and urinary T/E ratio in 4 healthy females and 4 healthy males (Karila et al., 1996). Volunteers were first administered 1.2 g/kg of EtOH in the morning,

for 4 h, provoking a 25% increase in serum T in females and a 7% increase in males compared to the baseline value. Their T/E ratio did not show an increase (T/E = 1.2 for males and T/E = 2.5 for females). More interesting results came from a higher dose. The volunteers were administered with 2 g/kg, and within 5 h the serum T concentration in women had increased by 25% (9.25 pM to 11.34 pM) but no significant change was noticed in men (2.93 pM to 2.95 pM), which the authors justify by the low number of volunteers (four) and high testosterone variation. As for urinary testosterone concentrations, these showed a 200 % increase in females and 25% in males. Urinary T/E ratio increased 500 % in women (0.7 to 4.4) but decreased in males (1.69 to 1.3). Serum concentrations of T in females are lower than in males, which makes a distinct difference even with small changes. This is supported by the fact that alcohol may change both the synthesis and metabolism of testosterone, since it stimulates steroid production by a direct mechanism in the weaker adrenal androgens which can then be converted to T by peripheral metabolism.

In another study, 1 g/kg was administered to 6 females and 6 males during 3 h (Seppenwoolde-Waasdorp et al., 1996). Urine was collected 1 day before the experiment took place, and then at timed intervals for 3 days. The time collection points are not described in the publication and it is hard to retrieve that information from the graphs presented. There was a significant increase in T/E in all subjects with females showing a 200 % increase from the pre-administration value (1.1 to 3.4), whereas the males had 50 % increase (0.8 to 1.2). In males, the increase in T/E was always followed by an increase in the concentration of excreted T, with either a decrease or slight increase in excreted E. In females, the increase in urinary T/E was not always followed by a consistent change on T and/or E.

The research group in the WADA accredited anti-doping laboratory in Germany (Cologne) investigated urinary T/E ratio in 1 male volunteer after ingestion of 2 g/kg, and there was 300 % increase (0.8 to 3.3) after 10.5 h of ingestion (Geyer et al., 1996).

The same group performed another study, with 5 females and 5 males (Mareck-Engelke et al., 1996). The initial experiment design was to administer 2 g/kg over a 4 h period in the morning. However, perhaps due to the early start, only 2 volunteers managed to drink the specified amount with the rest vomiting. Therefore, for these volunteers the amount of alcohol was

between 1.4 – 1.9 g/kg. Urine samples were collected every 4 h the day before the experiment took place; then after alcohol intake every 2 h up to 10 h; and finally every 4 h the day after the experiment. Average T/E values of females and males were compared in samples taken the day before the experiment, 8 h after alcohol administration and the day after. All the samples showed a highly significant increase due to alcohol with males increasing by 100 % (1.2 to 2.4) and females approximately 600 % (0.98 to 6.7).

Although there was an increase in urinary T/E in almost all volunteers, only females with a dose of 2 g/kg showed a urinary T/E > 4. The production of T differs in men and women, with men having the majority of T synthesised in the testes, women's T production comprises of ovarian and adrenal contributions (approximately 25 % each), and 50 % from peripheral interconversion from AD. It is also thought that, since women have less serum T than men any perturbance in that system will cause an increase in T.

All the above experiments were undertaken by administering a known amount of alcohol (maximum 2 g/kg) in a limited time. Although this is the best a researcher can do to mimic a social drinking session, it is possible to drink more than what the volunteers had in a longer period of time. Suffice to think of a Friday night pub-outing with your friends or work colleagues, where one could easily drink more than the volunteers were given, and for a longer period of time. Perhaps if one had ethical permission to do such study, changes in T/E ratio would be higher, or detectable in urine for a longer period of time.

A summary of the urinary T/E mean values found in the literature described above may be found in Table 1.7.

Table 1.7 Urinary T/E values after alcohol intake

Reference	Amount of EtOH	Number of subjects		Female		% increase	Male		% increase
		Female	Male	Before	After		Before	After	
(Falk et al., 1988)	0.5 – 1 g/kg	NA[6]	7	NA	NA	NA	1	1.13	13
	2 g/kg		4				1.14	1.52	33
(Karila et al., 1996)	1.2 g/kg	4	4	2.6	2.5	-4	1.69	1.3	-23
	2 g/kg	4	4	0.7	4.4	529	1.2	1.2	0
(Seppenwoolde-Waasdorp et al., 1996)	1 g/kg	6	6	1.1	3.4	209	0.8	1.9	138
(Geyer et al., 1996)	1.2 g/kg	NA	1	NA	NA	NA	0.8	3.3	313
(Mareck-Engelke et al., 1996)	1.4 – 1.9 g/kg	5	5	0.98	6.7	583	1.2	2.4	100

[6] NA stands for 'not available'

At the turn of the century, more studies were performed regarding the analysis of serum T in females and males.

In 2000, a study with teenagers (13-17 years old) arriving at the emergency department suffering from acute alcohol intoxication (AAI) was undertaken (0.8 g/L of EtOH) (Frias et al., 2000). Blood samples were collected between midnight and 3 a.m for both volunteers with AAI and for the control group (alcohol free). These samples showed increased serum T concentration in females contrasting with males, which showed a decrease when compared to the control group. This was believed to be due to the decrease of testicular T production through modifications of the NADH/NAD^+ ratio, and also the increase in β-endorphin which can decrease the testicular T production through their autocrine and paracrine effects on the testes. Similar results were obtained when the same type of study was perfomed in young adults between 20 – 27 years old (Frias et al., 2002).

In 2001, an analysis of premenopausal women (21 on oral contraceptives and 10 not) administered with 0.4 – 0.5 g/kg showed an significant increase in serum T, with levels increasing 300 % (Sarkola et al., 2001). Blood samples were collected hourly up to 7 h after alcohol ingestion, and a urine sample was taken 4 h after. The acute testosterone increase was more prominent in the women on oral contraceptives. Serum T concentration may be affected by changes in its synthesis in the adrenals and ovaries, by peripheral conversion from blood AD and DHEA or by a change in the catabolism in the liver. Since the increase of serum T was followed by a decrease in AD, and an increase in urinary AD and etiocholanolone, the authors postulate that the increase in serum T is due to alcohol's effect on the liver's metabolism of T. The rise in NADH to NAD^+ after alcohol intake may suppress the activities of the enzyme responsible for T's catabolism into AD.

A similar study by the same authors was undertaken in male volunteers after an intake of 0.5 g/kg (Sarkola and Eriksson, 2003). Similarly to what happens with women, serum T increases 19 % while AD decreases 22 %.

In 2006, 256 grams of alcohol was administered to healthy male volunteers for a period of 26 h starting with 20 g and then 10 g/h (Danel et al., 2006). Serum T levels were found to be significantly higher when compared to controls, with an increase of 26% (13.4 – 17 mmol/L),

and cortisol levels remained the same contrary to other findings following acute alcohol intoxication (Waltman et al., 1993). The authors present two hypotheses for the cortisol findings. The first is that the blood alcohol concentration (BAC) obtained was not enough to stimulate the HPA axis, hence there was no increase in cortisol. The second is that continuous administration over a 24 h period desensitises the HPA at the time it should be active. With the latter hypothesis, two different mechanisms need to be taken into account: the sensitivity to ethanol of the HPA, also having a circadian variation; and the repeated exposure to alcohol, which will induce tolerance. Therefore, the continuous administration of alcohol during the day may have desensitised the HPA, impeding the normal reactivation of the axis. Regarding serum T increased levels, it contrasts with previous studies where serum T decreased after acute alcohol intoxication (Frias et al., 2000, Frias et al., 2002). The authors postulate that such differences may have come with the different collection points since they collected blood samples every 2 h over 26 h, and in the other studies the collections were done between midnight and 3 am without taking into account nocturnal serum T levels.

A summary of the serum T concentrations mentioned previously may be found in Table 1.8. Serum T concentrations were performed by immunoassay.

A positive percentage increase is the increase over the total amount, and a negative percentage increase is a decrease over the total amount.

Table 1.8 Serum T concentration (ng/mL) after acute alcohol intoxication

Reference	Amount of EtOH	Number of subjects		Female		% increase	Male		% increase
		Female	Male	Before	After		Before	After	
(Karila et al., 1996)	1.2 g/kg	4	4	0.3	0.4	25	5.3	5.7	7
	2 g/kg	4	4	0.2	0.3	54	5.2	4.6	-11
(Frias et al., 2000)	0.8 g/L	16	11	0.45	2.21	391	4.7	3.7	-22
(Sarkola et al., 2001)	0.4 – 0.5 g/kg	21 OC[7]	NA	0.1	0.6	300	NA	NA	NA
		10 NOC[8]							
(Sarkola and Eriksson, 2003)	0.5 g/kg	NA	13	NA	NA	NA	3.9	4.6	19
(Danel et al., 2006)	256 g (0.5 – 0.7 g/L)	NA	11	NA	NA	NA	3.9	4.9	26

[7] Premenopausal women on oral contraceptive
[8] Premenopausal women not on oral contraceptive

1.9 Hypotheses

There is some concern in the sports anti-doping community as to the extent which ethanol ingestion affects the androgen balance and perturbs the T/E ratio (Falk et al., 1988, Karila et al., 1996, Frias et al., 2002).

This research project attempts to verify if:

1. Alcohol in serum can reach a concentration sufficient to displace T from its binding proteins such that serum T is raised, resulting in an increase in urinary T excretion and a raised urinary T/E ratio (protein binding – Chapter 2)

2. Two different doses of alcohol ingested within an hour in separate occasions will influence urinary T/E in females and males (excretion – Chapter 3)

3. Any increase in urinary T concentration is due to an increase in LH (production – Chapter 4)

4. Alcohol suppresses catabolism of T by enzyme inhibition, e.g. 17β-HSD2 (metabolism – Chapter 5)

5. Alcohol has an effect on hepatic metabolism of T (clearance – Chapter 6)

For the first hypothesis, an in vitro study with serum samples collected from volunteers was performed. For the remaining hypotheses, 20 volunteers were recruited (10 females and 10 males) and adminstered 4 and 8 units of alcohol in 1 h period, in two separate occasions. For the last hypotheses, this was done by investigating the effects of alcohol administration to hypogonadal men (do not produce T) receiving testosterone replacement therapy. This may help to clarify the effect of the liver to any transient increase in serum testosterone, as there should be a negligible contribution from testicular production.

Chapter 2 DISPLACEMENT OF TESTOSTERONE FROM ITS BINDING PROTEINS

2.1 Introduction

This chapter will verify *in-vitro*, if alcohol in serum can reach a concentration sufficient to displace T from its binding proteins such that serum T is raised, resulting in an increase in urinary T excretion and a raised urinary T/E ratio.

As mentioned in Chapter 1, section 1.5, in the blood stream, testosterone binds non-covalently to SHBG, albumin and to a much less extent to CBG (Wheeler, 1995). To summarise, of the circulating T in adult eugonadal men, approximately 45% is bound to SHBG, 50% to albumin and the other 5% circulates as free (Karagiannis and Harsoulis, 2005, Dunn et al., 1981). In eugonadal women, 66 % is bound to SHBG, 30 % to albumin and 4 % unbound. Testosterone has a higher affinity constant to SHBG than to albumin (1.6×10^9 M^{-1} and 3.6×10^4 M^{-1} respectively) but of lower capacity when compared to albumin due to the fact that the latter accounts for 60 % of the proteins present in plasma.

SHBG saturates when T reaches a concentration in the order of 10^{-5} M (Dunn et al., 1981) above which, the proportion of T binding to albumin can continue to rise. The percent of unbound T remains approximately the same since it would be necessary that albumin's binding sites would be saturated before there is an increase in free T (fT) (10^{-3}M) (Dunn et al., 1981).

2.1.1 Alcohol and SHBG

One of the first studies that investigated the effect of ethanol on SHBG consisted in a group of five male patients, maintained under an adequate nutritional program, which included three social drinkers and two alcoholics that had been abstinent for a week; and a second group of six male non-alcoholic patients (Gordon et al., 1976a). To both groups, an alcohol dose of 3 g/kg of body weight was administered for approximately a month.

Acute administration of alcohol (1-5 days) restrained episodic bursts of T, which caused an overall fall in the circulating T concentration. The plasma LH concentration did not decrease, indicating no suppression of secretion of this gonadotropin due to the decrease of episodic secretion of T in the first five days of alcohol administration, which may be linked with direct gonadal effect of alcohol. Prolonged administration (12-24 days) further reduced the mean

plasma concentration of T. Alcohol consumption also altered the peripheral metabolism of T, noticeable through the MCR increase. This might have been due to the decreased plasma binding capacity for T showed in this study in the non-alcoholic volunteers, as opposed to the increased concentration found in cirrhotic patients, and to the increased hepatic T A-ring reductase activity, which is likely to increase its hepatic extraction.

Abstinence studies in alcoholic male patients that had consumed at least 72 g/alcohol/day for the past five years (most had consumed 100-200 g/daily for 10-40 years) and were tested for T, fT, E2, DHT and SHBG showed normal values for T and DHT, E_2 and fT were above normal in 50% of the patients and SHBG was high in 60% of the cases with no distinction between cirrhotic and non-cirrhotic patients. (Lindholm et al., 1978).

Plasma steroid concentrations in 30 abstinent male alcoholic patients without liver failure have been reported with an alcohol intake of 150 g/day for the past 2 years uninterrupted for the month prior to the trial was investigated (Iturriaga et al., 1995). Although none suffered from liver failure, 9 had normal liver, 14 moderate alterations and 7 showed marked liver alterations. Testosterone, E_2, FSH, LH and SHBG were measured on admission and discharge. Alcoholic patients showed normal T values regardless of the presence of liver damage, but the concentrations of SHBG were remarkably raised in all alcoholic patients. SHBG values decreased after a short abstinence period without ever reaching normal values.

Low concentrations of T have been found in alcoholic cirrhotic patients (Bannister et al., 1987) and in cirrhotic patients (Gordon et al., 1975, Luppa et al., 2006) with the latter showing high concentrations of SHBG as well as E_2, the raised estrogen stimulating SHBG production.

In 21 non-cirrhotic male alcoholic patients (150 g/day for the past 2 years uninterrupted during the month previous to the trial) that had to remain abstinent for 12 weeks, measurement of the total plasma T concentrations were below the lower reference limit at admission but being within the reference range by day 10 (Iturriaga et al., 1999). The low concentration of fT and high concentration of LH are indicative of hypoandrogenism (deficiency of androgens in the body). Free T was low at admission reaching normal concentration by day 10, and correlating negatively with SHBG, which showed a 3-fold increase and still remained above the controls, slowly decreasing after abstinence. There is also a relative dissociation between SHBG and T,

since the values for fT remained the same even though SHBG concentration increased. Due to the high levels of SHBG provoked by alcohol intake it is possible that it might be used as a marker of excessive alcohol intake.

2.1.2 Determination of free T and total T

Equilibrium dialysis was the first method to be used in the determination of fT in serum (Sandberg and Slaunwhite, 1957) and will be described in more detail in the next section.

In this experiment, the authors used a portion of the radioactive testosterone (C^{14}), diluted it with a saline solution, put it in a cellulose casing (dialysis bag of semipermeable membrane) and equilibrated for 72 h. Small molecules, such as fT pass through the membrane from the bag into the buffer. Larger molecules and protein-bound ones are retained inside the bag. Solutions on both sides were extracted, hydrolysed and analysed. The percentage of unbound steroid was 7% for undiluted plasma, 9% if it was diluted 1:4 and 62% if diluted 1:40.

The same approach for an *in vitro* study was performed, by diluting with saline (1:5) a plasma sample with a known T concentration (Vermeulen et al., 1971). The sample was then dialysed for 15h at 37°C under shaking. The mixture was placed in 10 mL of saline with a tracer amount of tritiated T (^{3}H-T) in the presence of merthiolate used as a preservative. After reaching equilibrium, 0.5 mL of the portions were taken from the solutions inside and outside the dialysis tubing. The extracted portions were redissolved in 10 mL of scintillation fluid and the radioactivity determined in a liquid scintillation counter. The scintillation fluid converts that the radioactive energy to light which is detected by the β - scintillation counter thus measuring the ionizing radiation.

Other studies have estimated fT, and total T using the same type of technique (Vermeulen et al., 1999, Vermeulen et al., 1971, Vermeulen, 1973, Fritz et al., 2008). The total T concentration is calculated by analysing the dialysate, and fT by analysing the retentate.

The downsides of this type of experiments are the long equilibrium period, the fact that a high number of samples cannot be performed simultaneously,the dilution factor and the usage of knots or clamps in the dialysis bags which could loosen resulting in a leak, the extraction

process and working with radioactive substances which can be a health hazard (Wheeler, 1995).

Measurement of fT and total T by equilibrium dialysis can be time consuming but it is the most physiologically representative method and the most exact for estimating these concentrations. Care must be taken so that impurities do not bind to SHBG thus the labelled tracer T used must be highly purified hence the use of diluted serum samples as a precaution to avoid this situation (Vermeulen et al., 1999).

Recently, similar equilibrium dialysis investigations have been undertaken analysing the compounds using LC-MS/MS rather than immunoassay (Ji et al., 2005, Wan and Rehngren, 2006).

2.1.3 Equilibrium dialysis

Equilibrium dialysis is a separation technique for the study of molecular interactions. The results obtained in equilibrium conditions make it possible to study the low affinity interactions that would probably not be detected otherwise (Bioscience, 2002).

Its principle is to establish a state of equilibrium between serum containing a drug and a drug-free buffer (200-300 times the volume of the sample), after an incubation period at a set temperature. The chambers containing serum and buffer are separated by a semipermeable membrane with the appropriate molecular weight cut-off such that it will retain the receptor component of the sample which is what will bind the ligand (Thermo Scientific, 2008, Kwon, 2001).

The water molecules in the buffer (dialysate) are small enough to pass through the membrane and will diffuse into the serum chamber (retentate) because of the difference in osmotic pressure. This is called ‘volume shift’ and comprises of an increase in serum volume and a decrease in buffer volume. Please see the figure below for an illustrative description (Kwon, 2001).

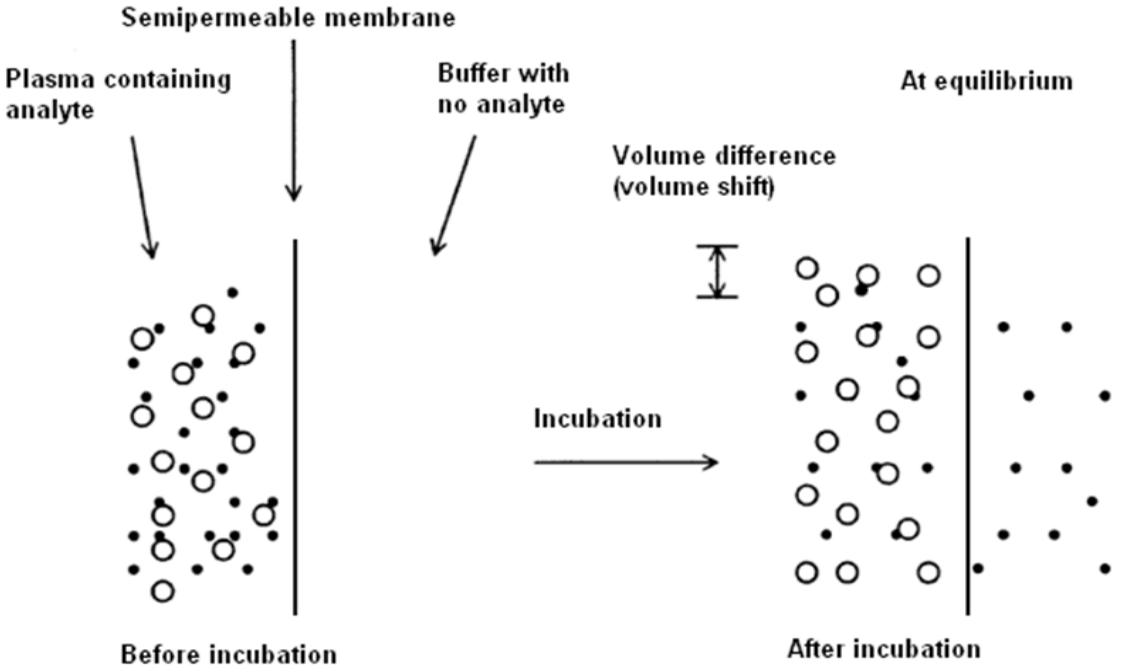


Figure 2.1 Equilibrium dialysis schematic. The analyte molecules (filled circles) not bound to proteins (empty circles) pass through the semipermeable membrane due to the osmotic pressure of the plasma containing the analyte. The volume changes from the buffer to the plasma side whilst reaching an equilibrium between the two chambers (Kwon, 2001)

In this project, initial experiments were performed using a dialysis cassette, Slide-A-Lyzer®, (Figure 2.2), which required 2 mL of serum. Another dialysis device in the shape of a tube, Float-A-Lyzer®, which required 1 mL of serum and less buffer volume thus decreasing serum dilution, was found in the market (Figure 2.3). No increase in sample volume was noticed with the Float-A-Lyzer®, so there was no need to adjust concentrations. Although the membrane in both devices is a very low protein binding membrane (regenerated cellulose in Slide-A-Lyzer® and cellulose ester in the Float-A-Lyzer®), the cylindrical geometry in the Float-A-Lyzer® is possibly the key factor to preventing sample dilution.

Figure 2.2 Dialysis cassette in a 400 mL beaker (Slide-A-Lyzer® cassette, by Thermo Fisher Scientific)

Figure 2.3 Dialysis tube in a 100 mL beaker (Float-A-Lyzer®, by Spectrum Labs)

The disadvantages of this technique are the length of time it takes the serum to reach equilibrium (18 h in previous published work (Fritz et al., 2008)), the need of a large volume of buffer and non specific binding to the membrane device.

A rapid equilibrium dialysis device (RED), by Thermo Scientific is now available and offers the possibility of reduced preparation and equilibration time. This device is based on a Teflon base plate holding up to 48 dialysis cells. Each cell consists of two side-by-side chambers separated by a vertical cylinder of dialysis membrane with a high membrane surface area-to-volume ratio (Cook et al., 2006, Waters et al., 2008). In the study by Cook *et al.*, the authors placed plasma containing T in one of the chamber and added the buffer to the other chamber. To determine non-specific binding a buffer sample containing the drug was added to both chambers and then incubated at 37° C under shaking at 100 rpm for 4 h. An aliquot was removed from each chamber and equal amounts of fresh plasma and buffer were added to respective incubated aliquots. Both mixtures (plasma and buffer) were precipitated with an organic/aqueous solution with 0.1% formic acid containing an internal standard mixture. After mixing and centrifugation, the supernant was transferred to a vial for LC-MS analysis.

Although this device solves the problem of the small number of samples that can undergo equilibrium dialysis in one day, it is also very expensive since the kit would cost a few hundred pounds. Each RED kit takes up to 48 samples, and with the number of experiments to be performed in this study (approximately 25) these costs were not considered justifiable.

2.1.4 Ultrafiltration

Another approach to test T's displacement involved ultrafiltration (Figure 2.4 and Figure 2.5) of a serum sample, and the analysis of the retentate and filtrate.

Figure 2.4 Ultrafiltration with semi-permeable membrane inside

Figure 2.5 Semi-permeable membrane (left) and ultrafiltration tube (right)

This technique is not an equilibrium and relies on the rapid separation of the free and bound fraction of a protein through centrifugation. It is similar to equilibrium dialysis in using a semi-permeable membrane to separate small molecules (free fraction) from large ones (protein bound) (Wheeler, 1995). Choosing an appropriate separation membrane allows the unbound fraction (fT) of low molecular weight to be separated (filtrate) from the protein-bound fraction (retentate). In our case, the separation membrane had a 30,000 molecular weight cut-off, since SHBG's molecular weight was 115,000, albumin's was 60,000, and CBG's was 50,000 Da.

With ultrafiltration, separation of the free and bound T can be performed much more rapidly, and it also avoids the problem of dialysis where T is naturally released from albumin as the system tends towards equilibrium.

However, ultrafiltration suffers from possible sources of error, including adsorption to the ultrafiltration device and protein leakage through the filter, which can cause misleading high concentrations of the apparent free fraction measured for strongly bound compounds (Eriksson et al., 2005), the volume of the filtrate may not be enough for the assay, constriction of membrane pores and the Donnan ion effect (Kwon, 2001).

2.1.5 Immunoassay

An immunoassay measures the concentration of a substance by means of an antibody/antigen complex. The antigen is normally the substance to be measured, and the antibody is generated by injecting the immunogen (in this case testosterone conjugated to a large protein such as albumin) into a host animal (usually a sheep or rabbit), stimulating the generation of the specific antibody which is then collected and used in the immunoassay.

There are two types of immunoassay: non-competitive and competitive, the latter being the one used for the detection of total T in serum. In the competitive fluoroimmunoassay described in the Materials and methods section of this chapter, there is competition between europium-labelled T and the sample T for anti-T antibodies. This is a specific type of assay called DELFIA (Delayed Enhanced Lanthanide Fluoroimmunoassay). The samples containing T inhibit the binding of the europium-labelled T to the antibody molecules. There is a blocking agent in the T assay buffer, which will facilitate the release of T from binding proteins in the sample; and a second antibody coated to the solid phase, which will bind the IgG-testosterone complex separating the antibody-bound and the free antigen. An enhancement solution will dissociate the europium ions from the labelled T into solution, forming fluorescent chelates which will then be measured. The fluorescence of each sample is inversely proportional to the concentration of T in the sample.

2.1.6 Gas-Chromatography Mass-Spectrometry

Chromatography is an analytical technique that separates the components of a mixture from each other.

Gas chromatography (GC) separates volatile compounds based on their relative partitioning between a stationary and mobile phase. In GC the mobile phase is an inert gas, and the stationary phase is coated onto the inside of a narrow capillary tube with an internal diameter of less than 1 millimetre (mm).

Since GC requires the analytes to be volatile it may be necessary to derivatise them. This involves a chemical reaction to turn the less volatile groups into more volatile ones.

The flow of the mobile phase or carrier gas (normally helium) is controlled by pressure regulators into a sample injection inlet heated to a temperature high enough to vaporise the compounds to be analysed at a defined temperature (Karasek and Clement, 1988). The GC column is attached to the injection port and the samples are introduced with a syringe through a flexible septum in the injection port at a temperature that is high enough to vaporise all the compounds. Sample introduction in capillary columns can be a delicate procedure because the open cross section of the capillary is very narrow, and the sample volume should be small so as not to overload the column. To prevent this type of situations, a split/splitless injector can be used (Rubinson and Rubinson, 2000c). Samples at a low concentration are injected in splitless mode and travel through the column with the carrier gas. In split mode, the carrier gas flows continuously through the inlet at a faster rate than the gas that flows through the column. The excess gas is vented and only a portion of the sample carries on to the column.

The most common ionisation modes in GC-MS are electron and chemical ionisation.

Chemical ionisation produces ions with little excess energy, producing a spectrum that is not so fragmented. It produces those ions by collision of the molecule with primary ions that are present in the source (Hoffmann and Stroobant, 2002a).

In electron ionisation, the ions are bombarded with a high-energy beam and producing more fragmented ions, so the molecular ion may not always be observed (Hoffmann and Stroobant, 2002a, Christian, 2004a).

The mass spectrometer (MS), is attached to the column exit (Figure 2.6) and monitors the sample components that elute from the column.

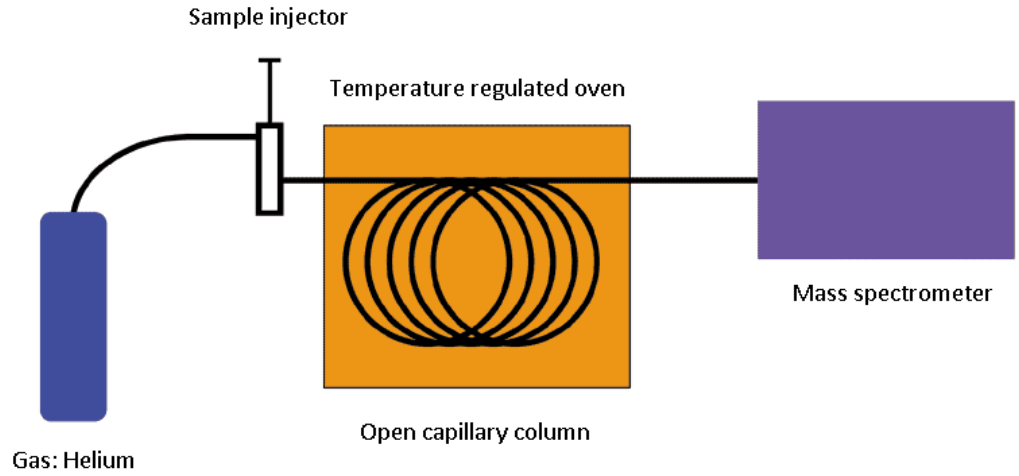


Figure 2.6 GC-MS schematic diagram

Mass-spectrometry is a powerful analytical tool used in the analysis of complex mixtures. Mass spectrometry analyses compounds in their gas-phase ions, for example by electron ionisation. Since most analytes are neutral, this is accomplished normally by bombarding the sample with high-energy electrons in an electron-ionisation source. The molecular ion undergoes fragmentation forming the product ions, which in turn may undergo fragmentation.

All the ions are separated according to their mass to charge ratio (m/z) and their detection proportional to their abundance (Hoffmann and Stroobant, 2002b). The fragments of a compound can thus tell us the structure of their precursor ion.

The mass-spectrometer used in the research was a quadrupole mass filter. The quadrupole consists of four rod shaped electrodes (Figure 2.7).

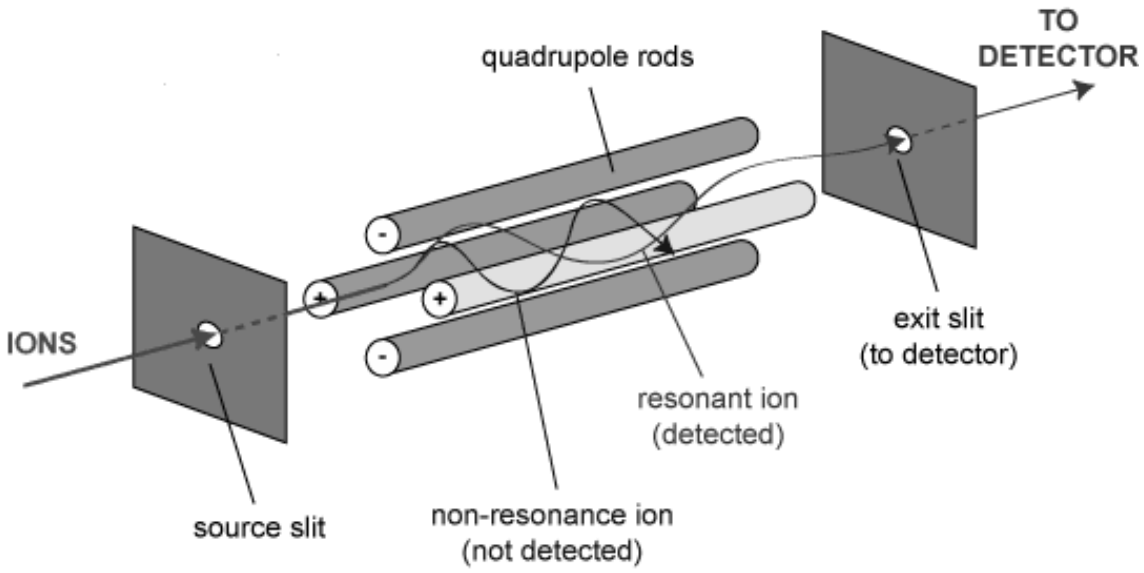


Figure 2.7 Quadropole mass filter (Bull, 2008)

A combination of alternating current (AC) and direct current (DC) fields filters the required ions by a m/z ratio using electric-field effects. A beam of ions passes through the middle of the rods, and since the voltage going through the rods is constant, separation of the different m/z ions is done because a radiofrequency AC is superimposed on the DC potential. Before the ions collide with the rod, the AC field drives them away in a complex oscillating path unique to each m/z ratio exiting through to the detector (Rubinson and Rubinson, 2000a).

2.1.7 Study untertaken

This chapter describes an *in vitro* study using serum spiked with alcohol, and monitoring the displacement of fT. Alcohol experiments' initial concentration was 350 mg% to mimic a sports case in which the athlete with a high urinary T/E had that blood alcohol concentration, estimated based on uncorroborated statements of the total amount of alcohol that the athlete had drunk.

A competitive immunoassay technique was employed to ascertain the amount of total T present in the retentate before and after the equilibrium dialysis, and GC-MS to measure fT in the dialysate.

2.2 Materials and methods

2.2.1 Materials

T and E were obtained from Promochem (Teddington, UK). Potassium carbonate, sodium sulphate, sodium chloride, sodium hydrogenphosphate anhydrous, potassium phosphate (analytical grade), hydrochloric acid, diethyl ether and methanol (HPLC grade) were all obtained from Fisher Scientific (Loughborough, UK). Potassium chloride and ammonium iodide (both analytical grade) were obtained from BDH Chemicals (Leicestershire, UK). MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide) and bamethan sulphate were obtained from Sigma-Aldrich (Germany), and ethanethiol from Sigma-Aldrich (Poole, UK). The tri-deuterated internal standards of d_3T, d_3E, $d_3$5α-AD and $d_3$5α-DHT, all labeled at positions 16, 16, 17α except for AD which is at position 19, were supplied by the National Analytical Reference Laboratory (Sidney, Australia). Vacutainers™ were acquired from BD Diagnostics (Oxford, UK). The Amicon® Ultra-4 centrifuge filter devices (4 mL) with a membrane of 30,000 MW were obtained from Millipore and the bovine serum albumin (RIA grade) was obtained from Sigma-Aldrich (Steinheim, Germany). The Slide-A-Lyzer® dialysis cassette was obtained from Thermo Scientific (Leicestershire, UK) and the Float-A-Lyzer® G2 from Spectrum Labs (Breda, Netherlands). The immunoassay auto-DELFIA® Testosterone kits were obtained from PerkinElmer® (Northwhich, UK), and the platewasher was a DELFIA® 1296-026.

2.2.2 Preparation of testosterone aqueous solution

2.2.2.1 Combined internal standard mixture

The laboratory's internal standard solution containing d_3T, amongst other deuterated compounds, was used.

Individual stock solutions of d_3T, d_3E, $d_3$5α-AD and $d_3$5α-DHT at a concentration of 100 μg/mL and bamethan at a concentration of 1 mg/mL were prepared in methanol. These were then

diluted in distilled water and methanol (50:50, v/v, in final solution) to make the combined internal standard mixture which contained the above compounds at the following concentrations: 1 μg/mL (d_3T and d_3E), 2 μg/mL ($d_3$5α-AD), 0.5 μg/mL ($d_3$5α-DHT) and 5 μg/mL (bamethan). In a 2 mL solution, spiked with 100 μL of internal standard mixture, the concentrations would have been 50 ng/mL for d_3T and d_3E, 100 ng/mL ($d_3$5α-AD), 25 ng/mL ($d_3$5α-DHT) and 250 ng/mL (bamethan)

A dilution (1:500) of the combined internal standard mixture was made to have d_3T at 0.10 ng/mL for the experiment involving aqueous T solution with SHBG.

2.2.2.2 Testosterone stock solution

Testosterone stock solutions were prepared in water:methanol 7:3, v/v, and then diluted to the required concentrations with distilled water.

The samples were labelled as JLV standing for the author's initials, followed by a chronological number.

2.2.2.3 Experimental design

2.2.2.3.1 Testosterone aqueous solution at room temperature

To determine the time it takes to achieve equilibrium, and in line with previous *in vitro* experiments analysing male serum (Sandberg and Slaunwhite, 1957), equilibrium dialysis experiments were initially performed with testosterone in aqueous solution, at room temperature, at three different concentrations: 22.5, 45 and 375 ng/mL. These concentrations were chosen in order to obtain 0.15 ng/mL, 0.3 ng/mL and 2.5 ng/mL in the dialysate.

Each solution (2 mL) was placed inside an individual dialysis cassette, which in turn was put in 300 mL of deionised water in a beaker and left under stirring at room temperature (RT), covered

with tin foil to minimise evaporation. Two mL was taken from the beaker every 2 h until 10 h for all the samples to determine the time it would take to reach equilibrium. The dialysate samples were extracted and analysed by GC-MS.

2.2.2.3.2 Testosterone aqueous solution with alcohol at room temperature

To check if the presence of alcohol affected the extraction procedure, 350 mg% of ethanol (44 μL) was added to 10 mL of a 4.5 ng/mL aqueous solution of testosterone (JLV074), left standing for 30 min at room temperature and then extracted and analysed by GC-MS.

2.2.2.3.3 Testosterone & SHBG aqueous solution with alcohol at room temperature

Testosterone (2 mL) at a concentration of 7.5 ng/mL (SHBG saturates at this concentration) in aqueous solution along with 6.44 μg of SHBG was placed inside a dialysis cassette, which was then put in 300 mL of deionised water and left under stirring at room temperature for 24 h (6.44 μg would produce an SHBG concentration of 28 nM, which is the plasma concentration reported previously (Dunn et al., 1981)). After 24 h, 8.8 μL of ethanol was added to the sample (giving 350 mg% of ethanol) which was then left under stirring for another 24 h, finally 10 mL of dialysate was taken, extracted and analysed under GC-MS (JLV076).

2.2.2.4 Extraction procedure (2 mL extracts):

For the extraction, 2 mL of water blank and four calibration standards (0.1, 0.2, 0.5 and 1 ng/mL) in water were placed in a 20 mL glass tube along with 20 μL of internal standard (0.1 ng/mL of internal standard in 2 mL of solution). Potassium carbonate was added to each sample (100 mg) followed by 5 mL of diethyl ether prior to vortex mixing for 30 seconds, with the organic layer subsequently removed and placed into a 20 mL glass tube.

Anhydrous sodium sulphate was then added whilst vortexing and the sample allowed to stand for 2 min. The samples were then centrifuged at 100 *g* for 5 min before decanting off the organic phase into a 10 mL glass tube. The test tubes used for liquid/liquid extraction (now containing sodium sulphate) were rinsed with 2 mL of diethyl ether, which was then pooled with the previous fraction. The solvent was then evaporated to dryness under nitrogen at 30 °C ± 5 °C and the dried extracts placed a desiccator for at least 30 min.

The test tubes were flushed with nitrogen and the steroid extracts converted to trimethyl silyl derivatives under forcing conditions to ensure that complete enol as well as ether derivatisation occurred, to achieve this MSTFA:NH_4I:ethanethiol (20 μL) was added. Samples were heated (60 °C ± 5 °C) for 20 min, and then allowed to cool. Finally 20 μL of dodecane was added and the samples vortexed before transfering into an autosampler vial and analysed by GC-MS.

2.2.2.5 Extraction procedure (10 mL extracts)

The free concentration of testosterone in serum is less than 2.5 ng/mL (assuming normal T concentration in males as ~7 ng/mL, then fT would be ~0.14 ng/mL). In the experiments with serum,the volume of dialysate taken was changed to 10 mL rather than 2 mL to increase the amount of analyte analysed. The volume of internal standard was also increased five times (100 μL).

For the extraction, 2 mL of water blank and four calibration standards (0.1, 0.2, 0.5 and 1 ng/mL) were extracted as above (section 2.2.2.4) for quantification. To 10 mL of dialysate, 100 μL of internal standard was added.

Potassium carbonate was then added to each 10 mL sample (300 mg). Liquid/liquid extraction was performed with 5 mL of diethyl ether, which was added prior to vortex mixing for 30 seconds with the organic layer subsequently removed into a 20 mL glass tube. This extraction was then repeated a further two times with the organic layer being pooled.

The solvent was then evaporated to dryness under nitrogen at 30 °C ±5 °C and put into the desiccator for at least 30 min and the samples were derivatised and analysed by GC-MS.

2.2.2.6 GC-MS conditions

Methods validated by the Drug Control Centre for urinalysis were used, but the urine replaced by water. The GC column was HP-1 (methylsiloxane) (25 m, 0.2 mm, 0.11 μm) from Agilent, used for both methods. For the calibrants the laboratory method 'TETCAL' was used, consisting of an initial oven temperature of 180 °C for 1 min, ramping at 8 °C/min until it reached 220 °C, and then 3 °C/min to 244°C. The total run time was 14 min.

For the samples, the method 'TETSAMP' with an initial oven temperature of 180 °C for 1 min, ramping at 8 °C/min to 220 °C, then 3°C/min to 250 °C, and then 14°C/min to 280 °C held for 5 min. The total run time was 23.14 min.

The method was run in selective ion monitoring (SIM) mode and the ions monitored were 432.3/435.3 for T/d_3T. The ion source used was electron ionization and T's retention time was 13.1 ± 0.15 min..

2.2.2.7 Immunoassay serum analysis

The DELFIA® Testosterone immunoassay kit was used following the instructions protocol provided with the kit. This assay has been validated by the manufacturer according to the Food and Drug Administration regulation, and the analytical sensitivity was determined to be better than 0.09 ng/mL.

2.2.3 Volunteer study and sample collection

Ethical approval was sought from the King's College London Ethics Committee and given for the collection of 10 mL of blood (2 teaspoons) from male volunteers from a suitable arm vein. Recruitment was done via King's College London (KCL) mailing list. The blood collection was performed by a trained phlebotomist and took between 5-10 minutes.

To maintain anonymity of the volunteers, a numeric code was given to each volunteer's sample. The samples were coded and kept in a restricted access freezer within the Drug Control Centre (DCC), an evidential laboratory, which itself has restricted access. Only the study's investigators had access to the volunteers's details and the consent forms were kept in a box inside a locker.

2.2.4 Experimental design with serum using equilibrium dialysis

2.2.4.1 Sample preparation

Each sample of blood was collected in 8 mL Vacutainers™ and then left to clot for 30 minutes (min), after which it was centrifuged at 1000 *g* for 10 min. The supernatant (serum) was transferred into a polypropylene tube and stored at -20°C, until needed, at which point rapid thawing was performed employing a water bath at 37°C.

2.2.4.1.1 Serum testosterone equilibrium at room temperature

A volume of 2 mL of serum was placed inside a dialysis cassette, which was then immersed in 300 mL of phosphate buffer saline (PBS) at a pH of 7.4 (Sambrook, 1989) and left under stirring for 24 h at room temperature. The PBS solution was prepared to yield the concentrations of sodium chloride at 137 mM, potassium chloride at 2.7 mM, potassium phosphate at 2 mM and sodium hydrogenphosphate anhydrous at 10 mM. The serum was removed from the dialysis cassette and analysed by immunoassay (IA) (JLV113).

2.2.4.1.2 Serum testosterone with alcohol at room temperature

A volume of 2 mL of serum was placed inside a dialysis cassette which was then immersed in 300 mL of PBS with a pH of 7.4 (same as serum) and left under stirring for 24 h at room temperature. After that time, 8.8 μL (350 mg%) of ethanol was added to the sample and 1.33 mL to the PBS and left under stirring for another 24 h after which the serum was removed and analysed by IA (JLV083).

2.2.4.1.3 Total testosterone measurement by immunoassay

Using a DELFIA Testosterone kit it was necessary to reconstitute the standards, the tracer and the antiserum by leaving them to dissolve for 30 min in 1.7 mL of distilled water. The tracer and antiserum were then diluted according to the manufacturer's instructions. The necessary strips were washed once in the DELFIA platewash with a wash solution containing tris-HCl buffered salt (pH 7.8) with Tween 20 and the standards and unknowns were added (25 μL) to it followed by the tracer and antiserum solutions (100 μL). The strips were left incubating (slow shaking) for 2 h at room temperature and afterwards washed four times with the wash solution. The enhancement solution (200 μL) provided with the kit was added and the strips were left for 5 min slowly shaking. The concentration of testosterone was then measuring using a Victor 3 multilabel counter (Perkin-Elmer®).

2.2.4.1.4 Serum & alcohol immunoassay

To verify if the presence of alcohol had any influence in the serum immunoassay, 5 μL of EtOH (approximately 350 mg%) were added to 1 mL of serum (JLV097) and analysed by immunoassay against the control (JLV 084).

2.2.4.1.5 Subsequent experimental changes

To assess the influence of some of the major experimental variables the following changes were made to the previously described experimental procedure with serum:

1. Temperature raised to 37 °C rather than room temperature (JLV130)

2. Double the amount of ethanol (JLV107)

3. Variation in the volume of buffer from 300 to 250 mL (JLV105)

4. Variation in dialysis device by using a tube dialysis (Float-A-Lyzer) instead of cassette: 1 mL of serum in 54 mL of buffer (1:54 dilution rather than 1:150) (JLV104), at room temperature

5. Variation in dialysis device, (Float-A-Lyzer) and temperature at 37 °C (JLV158)

2.2.5 Experimental design with serum using ultrafiltration

Serum samples were obtained as detailed in 2.2.3.

Four aliquots of 1 mL each were put into 2 mL plastic tubes. The first sample was used as a control (JLV145). Ethanol was added to the remaining samples in the following concentrations: 350 mg% (JLV146), 700 mg% (JLV147) and 1400 mg% (JLV148). The four samples were then placed on a rotatory mixer, for 2 h 30 min in a 37°C incubator. Each sample was put into an ultracentrifugation device and centrifuged in a swinging bucket rotor at 4000 *g*.

The ultracentrifugation devices were rinsed with a 10 % BSA/PBS solution, and 50 μL of a 1 % BSA/PBS solution was placed at the bottom of each tube to prevent nonspecific binding to the walls of the tube or to the ultrafiltration membrane. The tubes were weighed before and after the sample was put in, and by assuming the serum density to be 1 g/mL, the volume of filtrate and retentate could be determined.

The retentate was then reconstituted in PBS so as not to be as concentrated and both retentate, filtrate and control were analysed by immunoassay for T.

2.2.6 Intra-assay precision

For the intra-assay variability experiment, six aliquots (2 mL) of dialysate were pipetted out of each beaker (section 2.2.2.3.1), extracted and analysed by GC-MS (Appendix 8.2).

2.3 Results and discussion

2.3.1 Lower limit of quantification

The lower limit of quantification (LLOQ) was defined as the concentration at which the signal to noise ratio (S/N) was 10:1 in aqueous solution. For T, the LLOQ was 0.1 ng/mL (Figure 2.8).

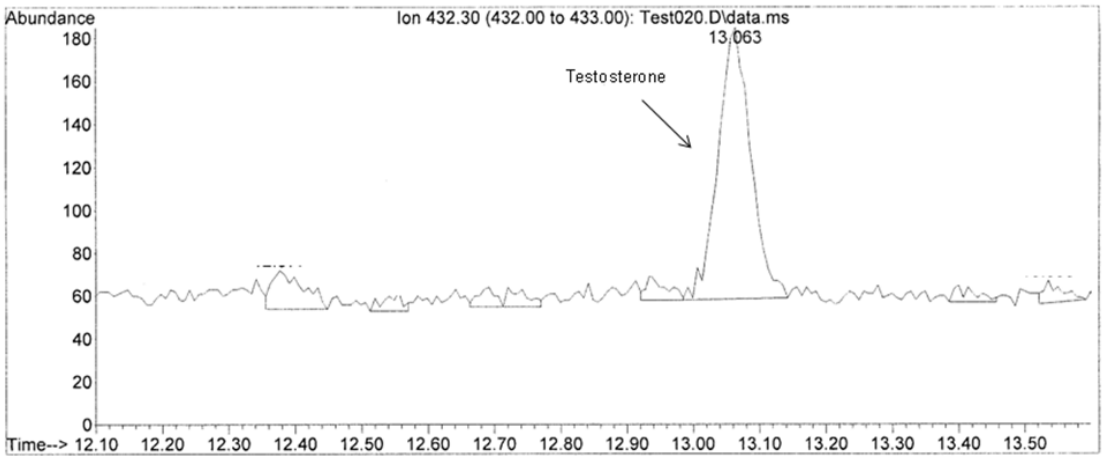


Figure 2.8 Lower limit of detection for testosterone at 0.1 ng/mL

2.3.2 Testosterone aqueous solution

2.3.2.1 Intra-assay precision

Equilibrium dialysis was performed using three different concentrations of testosterone in aqueous solution to ascertain the equilibrium time, and to verify if the concentration had any influence on the equilibration time (Table 2.1).

Table 2.1 Initial & expected concentrations for equilibrium dialysis (initial retentate volume = 2 mL, dialysate volume = 300 mL)

Concentration in retentate (ng/mL)	Expected concentration in the dialysate (ng/mL)
22.5	0.15
45	0.3
375	2.5

In order to be able to better interpret the equilibrium time course data, a separate experiment to assess assay variability was conducted and the data obtained shown below.

The data are labelled using the prefix JLV for each sample, and results are shown in Table 2.2, Table 2.3 and Table 2.4. These numbers are assigned in chronological order but for clarity the results of the intra-assay variability experiment are presented before those of the equilibrium dialysis data.

Table 2.2 Intra-assay variability for an expected dialysate T of 0.15 ng/mL

Sample	Concentration (ng/mL)
JLV034	0.07
JLV035	0.09
JLV036	0.08
JLV037	0.09
JLV038	0.09
JLV039	0.08
Average	**0.08**
SD	**0.01**
RSD (%)	**9.8**

Table 2.3 Intra-assay variability for an expected dialysate T of 0.30 ng/mL

Sample	Concentration (ng/mL)
JLV040	0.42
JLV041	0.44
JLV042	0.48
JLV043	0.41
JLV044	0.42
JLV045	0.45
Average	**0.44**
SD	**0.03**
RSD (%)	**5.9**

Table 2.4 Intra-assay variability for an expected dialysate T of 2.5 ng/mL

Sample	Concentration (ng/mL)
JLV046	2.93
JLV047	2.98
JLV048	2.8
JLV049	3.03
JLV050	3.12
JLV051	3.08
Average	**2.99**
SD	**0.12**
RSD (%)	**3.9**

The intra-assay variability was found to be less than 10 %.

Regarding the time course experiment using initial retentate T concentrations of 22.5, 40 and 375 ng/mL, dialysate samples (2 mL) were taken every 2 h starting at 0 h until 10 h and analysed by GC-MS (Appendix 8.3). Results are shown in Table 2.5, Table 2.6 and Table 2.7.

Table 2.5 Time course of equilibrium dialysis. Initial retentate T concentration = 22.5 ng/mL. Expected dialysate T concentration 0.15 ng/mL

Sample	Time (h)	T dialysate concentration (ng/mL)
JLV 010	0	0.01
JLV 011	2	0.08
JLV 012	4	0.09
JLV 013	6	0.09
JLV 014	8	0.08
JLV 015	10	0.1

Table 2.6 Time course of equilibrium dialysis. Initial retentate T concentration = 40 ng/mL. Expected dialysate T concentration 0.30 ng/mL

Sample	Time (h)	T dialysate concentration (ng/mL)
JLV 016	0	0
JLV 017	2	0.25
JLV 018	4	0.34
JLV 019	6	0.28
JLV 020	8	0.30
JLV 021	10	0.29

Table 2.7 Time course of equilibrium dialysis. Initial retentate T concentration = 375 ng/mL. Expected dialysate T concentration 2.50 ng/mL

Sample	Time (h)	T dialysate concentration (ng/mL)
JLV 022	0	0.03
JLV 023	2	1.91
JLV 024	4	1.89
JLV 025	6	1.94
JLV 026	8	1.90
JLV 027	10	1.97

For the experiment where the final dialysate T concentration should reach 0.15 ng/mL, at 10 h, 67 % of this expected concentration had been reached, notwithstanding experimental variability for the sample (Table 2.5, measured dialysate T = 0.1 ng/mL) which is to be expected given that most of the concentrations were less than the assay's LLOQ of 0.1 ng/mL.

In the experiment where the final dialysate T concentration should reach 0.30 ng/mL, at 2 h, 83 % of this expected concentration had been reached. Thus the same percentage (67 %) as in the previous experiment, where the dialysate T concentration should reach 0.15 ng/mL, must have been reached before 2 h rather than 10 h. At 10 h, 97 % of the expected T dialysate concentration was obtained (Table 2.6).

In the experiment where the final dialysate T concentration should reach 2.5 ng/mL, at 2 h, 76 % had been reached and at 10 h, 79%.

Graphs were plotted (Figure 2.9, Figure 2.10 and Figure 2.11) using the data from Table 2.5, Table 2.6 and Table 2.7, describing the expected asymptotic behaviour, and ± 2 standard deviation (SD) plotted in the graph to indicate which concentration changes could be considered due to the dialysis rather than assay variability.

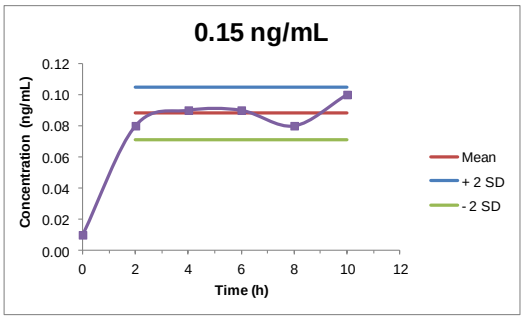


Figure 2.9 T concentration against time (expected maximum of 0.15 ng/mL) with ±2 SD

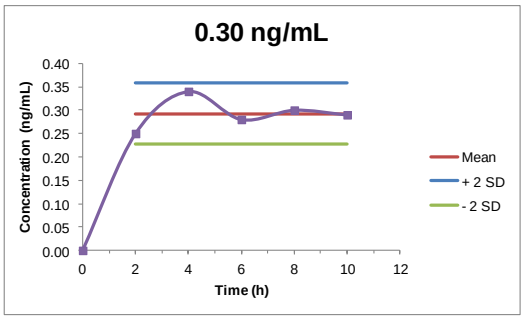


Figure 2.10 T concentration against time (expected maximum of 0.30 ng/mL) with ±2 SD

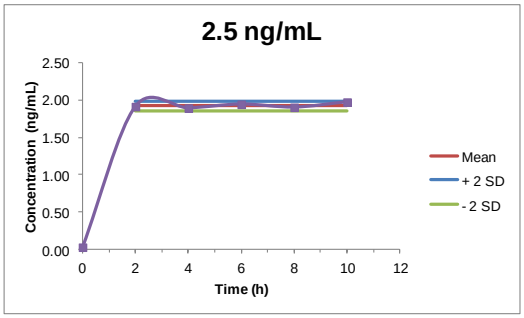


Figure 2.11 T concentration against time (expected maximum 2.5 ng/mL) with ±2 SD

Equilibrium was reached at 2 h for an expected dialysate T concentration of 0.15 and 0.30 ng/mL, and at 4 h for an expected dialysate T concentration of 2.5 ng/mL. This shows that equilibrium of fT in an aqueous solution is fairly rapid and does not appear to be concentration dependent.

However, because this experiment was performed with fT in an aqueous solution as the retentate, it was not possible to be sure if serum T would behave in the same way with equilibration being achieved within 2 h, or even 4 h.

The next logical step would have been to investigate the time course equilibrium with serum, by analysing the dialysate, in order to check if the rate of displacement of T from binding proteins was of the same time order as the fT in the aqueous solution. However, the lack of sensitivity of the assay made this impossible (since the serum T concentration would be in the order of only 7 ng/mL giving a dialysate concentration of 0.046 ng/mL). Furthermore since fT comprises 1 % of total T, the concentration would be even less if displacement of binding does not occur. Also,

multiple analysis of the serum retentate would not have been possible due to sample handling. The dialysis cassette is not designed for repeated sampling since it has a fragile membrane that is easily torn.

Previous published work used an equilibration time of 18h, therefore, from a practical point of view, we thought that 24 h would be a sufficient time for serum T binding to reach equilibrium (Fritz et al., 2008).

2.3.2.2 Testosterone aqueous solution with alcohol at room temperature

The calibration plot analysis by GC-MS (Appendix 8.4) of the extract containing 4.5 ng/mL of T and 350 mg% of EtOH showed that the extract had 4.52 ng/mL T (JLV074), therefore showing that EtOH does not affect the extraction procedure.

2.3.2.3 Testosterone & SHBG aqueous solution at room temperature

The apparent concentration obtained for JLV076 was of 0.03 ng/mL (Appendix 8.5) when it was expected to be 0.0005 ng/mL. However, the LLOQ of the GC-MS methods is 0.1 ng/mL so the apparent difference between the obtained and the theoretical concentrations is probably simply due to analytical error. In fact, the internal standard (d_3T) contains about 0.3 % of T. Since a d_3T concentration of 0.1 ng/mL was used, this would contribute 0.0003 ng/mL of T, i.e. 60 % of the expected concentration, which was not corrected in the calibration. Another possible reason for this increased measured T is that the SHBG was not given enough time to equilibrate with the T present in solution, and therefore may have diffused before binding to SHBG took place.

Since the same is applicable to T in serum, the dialysate was not analysed by GC-MS in the experiments that followed. The theoretical calculations follow:

If we assume a total T concentration in serum of 22.5 nmol/L (6.5 ng/mL) the maximum concentration in 300 mL, so that the concentration in 2 mL is the same as in the 300 mL, will be 0.31 nmol/L (0.09 ng/mL).

Another pertinent question would be, is the fT in the smaller compartment (2 mL) more or less than 0.09 ng/mL? If it is less, then it will not be dissociated from that protein. This can be calculated theoretically as follows:

T bound to albumin:

[Albumin] = 0.56 mmol/L (33.6 mg/mL)

$K_a = 3.6 \times 10^4\ M^{-1}$

$K_a = ([albumin] \times [T]) / [bound\text{-}T]$

Free T is estimated to be 1 % and hence total T is approximately the same as bound T at 22.5 nmol/L (6.5 ng/mL22.5 nmol/L). Since about 50 % (Dunn et al., 1981) is bound to albumin at a concentration of 11.25 nmol/L (3.2 ng/mL), the maximum of bound T that could be dissociated from albumin is 0.57 nmol/L (0.16 ng/mL).

T bound to SHBG:

[SHBG] = 28 nM = 3.22×10^{-6} g/mL = 3220 ng/mL

$K_a = 1.6 \times 10^9\ M^{-1}$

$K_a = ([SHBG] \times [T]) / [bound\text{-}T]$

Assuming the same as above, total T will be about the same as bound T, and approximately 44 % (Dunn et al., 1981) is bound to SHBG accounting for 10 nM (2.9 ng/mL), the T concentration that will be displaced form SHBG is 0.22 nmol/L (0.06 ng/mL).

These calculations also show us that it is not very likely that T is dissociated from SHBG by equilibrium dialysis but that the T found will probably come from the natural dissociation of T from albumin that equilibrates between the two compartments on either side of the dialysis membrane with time. Nevertheless, it was considered necessary to confirm these deductions experimentally.

2.3.3 Serum testosterone experiments using equilibrium dialysis

2.3.3.1 Serum testosterone equilibrium at room temperature

Using 24 h as the time for an aqueous T solution to equilibrate in the cassette with no protein binding, an experiment with serum was necessary to verify how much T was displaced (and equilibrated) from the retentate to the dialysate. Since the concentration of fT in the dialysate would be too small to be determined by GC-MS, the retentate only was analysed by immunoassay and compared to a control.

The immunoassay results for the control (JLV101) were 20.2 nmol/L (5.8 ng/mL), and for the sample (JLV113) 11.8 nmol/L (3.4 ng/mL) (Appendix 8.6). However, the final volume was not 2 mL but had increased 2.9 mL, due to diffusion of buffer into the dialysis cell, so after adjusting the concentration to 2 mL it would have 17.1 nmol/L (4.9 ng/mL), meaning that there was a 15 % displacement of T from the retentate to the dialysate (Table 2.8).

As described in section 2.2.4.1.4, a 1 mL serum sample was spiked with 5 μL of EtOH to verify if it had any influence in the immunoassay. The control (JLV084) was 13.4 nmol/L (3.9 ng/mL) and the sample (JLV097) was 13.1 nmol/L (3.8 ng/mL), therefore we can conclude that EtOH does not affect the immunoassay analysis.

2.3.3.2 Serum testosterone with 350 mg% alcohol at room temperature

After 24 h of dialysis, and immediately before the addition of 350 mg% of EtOH, a small sample (~70 μL) of serum was taken from the dialysis cassette. Both the 24 h dialysed serum (JLV091) and the serum taken from the dialysis cassette 24 h after the addition of alcohol were measured (JLV096) and compared against its control (JLV090). The final volume of sample taken from the dialysis cassette was 2.8 mL.

The concentration of T for JLV091 was 9.1 nmol/L (2.6 ng/mL) and for JLV096 was 6.5 nmol/L (1.9 ng/mL) (Appendix 8.7). Adjusting both concentrations to a 2 mL sample serum, one would have 12.7 nmol/L (3.7 ng/mL) for the serum sample before the addition of EtOH, and 9.1 nmol/L (2.6 ng/mL) after the addition (assuming the final volume of 2.8 mL at 48 h was the same as at 24 h). The control was at a concentration of 19.7 nmol/L (5.7 ng/mL) therefore there was a 35 %

displacement from the retentate to the dialysate, before the addition of EtOH and a 54 % displacement after its addition (Table 2.8). It was not possible to be sure if this displacement was due to the addition of EtOH causing a displacement of T (~ 50%), or if it was the natural occurring displacement from albumin. To verify this, further experiments were performed.

2.3.3.3 Serum testosterone with 350 mg% alcohol at 37 °C

The same experiment as above was repeated, but using a 37 °C incubator and a positive control. The serum to which EtOH was added (350 mg%) was JLV130, and the positive control JLV129. After 48 h both samples were analysed by immunoassay and compared to the negative control (JLV116). The negative control had a concentration of 19.7 nmol/L (5.7 ng/mL), the positive control 3.8 nmol/L (1.1 ng/mL) and the sample to which EtOH had been added was 3.7 nmol/L (1.1 ng/mL) (Appendix 8.8). The final volume of the positive control was 3.9 mL and for the sample 3.2 mL. Therefore the adjusted total T concentrations were 7.4 nmol/L (2.1 ng/mL) for JLV129 (62 % displacement), and 5.9 nmol/L (1.7 ng/mL) (70 % displacement) (Table 2.8). Since the displacement of the sample to which EtOH was added is not much greater than the positive control's displacement, it does not seem likely that the displacement of T from binding proteins is due to alcohol. However, this displacement is higher than the approximately 50 % expected from the natural displacement that occurs from albumin. Alcohol does not have an effect on the displacement, but temperature does affect the affinity constant and/or the equilibrium time.

2.3.3.4 Serum testosterone with alcohol 700 mg% at room temperature

In order to verify if an increase in EtOH would force the displacement of T from its binding proteins, the same type of experiment was performed at room temperature but at the end of 24 h, 700 mg% of EtOH were added instead of the usual 350 %mg (Appendix 8.9). The negative control was JLV101 with a concentration of 20.2 nmol/L (5.8 ng/mL) (Appendix 8.8), and the serum sample JLV107 with a total T concentration of 7.2 nmol/L (2.1 ng/mL) (Appendix 8.8). The final volume of the serum sample was 3.1 mL and the adjusted concentration

11.2 nmol/L (3.2 ng/mL), showing a displacement in T of 45% (Table 2.8), hence the increase in alcohol does not have an influence in the displacement of T from its binding proteins.

2.3.3.5 Serum testosterone in a 250 mL volume of buffer with 350 mg% of EtOH at room temperature

Using a smaller beaker, the same experiment was performed but with 250 mL of PBS rather than 300 mL, and with an EtOH concentration of 350 mg% being added at the end of 24 h.

The negative control (JLV101) had a concentration of 20.2 nmol/L (5.8 ng/mL) (Appendix 8.10), and the sample (JLV105) was 6.5 nmol/L (1.9 ng/mL). The final volume of the sample was found to be 3.1 mL and therefore the adjusted total T concentration for 2 mL was 10.1 nmol/L (2.9 ng/mL), which implies a displacement of 50 % (Table 2.8). There isn't a significant difference between using 250 mL buffer and 300 mL (section 2.3.3.2).

2.3.3.6 Serum testosterone in tube dialysis at room temperature with 350 mg% of EtOH

After the above experiments were performed, the Float-A-Lyzer device became available, a device where the dialysate volume should remain constant, and the same type of experiment was performed but using 1 mL of serum in 54 mL of buffer. The control (JLV101) had a concentration of 20.1 nmol/L (5.8 ng/mL) and the sample (JLV106) was 12.3 nmol/L (3.5 ng/mL) (Appendix 8.11). The displacement of T from its binding proteins was 40 % (Table 2.8) showing no significant difference between this dialysis device at room temperature and the dialysis cassette (section 2.3.3.2).

2.3.3.7 Serum testosterone in tube dialysis at 37 °C with 350 mg% of EtOH

The same experiment as above was performed at 37 °C with a positive control. The negative control (JLV118) had a concentration of 20.1 nmol/L (5.8 ng/mL). After 48 h the positive control (JLV157) was 4.1 nmol/L (1.2 ng/mL), and the sample (JLV158) 4.0 nmol/L (1.2 ng/mL) (Appendix 8.12). There was no need to adjust the concentration to the initial volume since the

final volume was approximately the same as the initial one. The displacement of the positive to the negative control, and from the sample to the negative control was 80 % (Table 2.8).

It was observed that this device allows a better retention of the sample volume, thus not being necessary to adjust the concentrations to the final volume in the dialysis device. Comparing this experiment with the previous one (section 2.3.3.6) performed at room temperature there was a higher displacement, but when comparing the positive control with the sample, alcohol does not appear to influence the displacement.

When comparing the displacement values from this experiment and its analogue with the dialysis cassette (section 2.3.3.3) we can verify that both had a similar type of displacement (80 % and 70 % respectively). This is also true for the same experiments at room temperature. The tube dialysis experiment at room temperature (section 2.3.3.6) showed a displacement of 40 % and the dialysis cassette one a displacement of 54%.

According to these results, temperature does have an influence in the displacement of T from its binding proteins but it is still not possible to know if this displacement is due to the influence of temperature on the affinity constant of the binding proteins, or on the equilibrium time.

2.3.3.8 Summary of equilibrium dialysis and immunoassay results

The results for the equilibrium dialysis involving the above experiments (2.3.3.1 to 2.3.3.7) can be found in Table 2.8. The (+) indicates the positive control.

Table 2.8 Summary of results from serum equilibrium dialysis experiments

Experiment type	IA (negative control)	IA (sample)	V of sample (mL)	Adjusted [T] to 2 mL	% of displacement between sample & control
24h equilibrium	JLV101 = 20.2 nmol/L (5.8 ng/mL)	JLV113 = 11.8 nmol/L (3.4 ng/mL)	2.9	17.1 nmol/L (4.9 ng/mL)	15
Serum + 350 mg % EtOH at RT	JLV090 = 19.7 nmol/L (5.7 ng/mL)	JLV091 = 9.1 nmol/L (2.6 ng/mL)	2.8	12.7 nmol/L (3.7 ng/mL)	35
		JLV096 = 6.5 nmol/L (1.9 ng/mL)		9.1 nmol/L (2.6 ng/mL)	54
Serum + 350 mg % EtOH at 37°C	JLV116 = 19.7 nmol/L (5.7 ng/mL)	(+) JLV129 = 3.8 nmol/L (1.1 ng/mL)	3.9	7.4 nmol/L (2.1 ng/mL)	62
		JLV130 = 3.7 nmol/L (1.1 ng/mL)	3.2	5.9 nmol/L (1.7 ng/mL)	70
Serum + 700 mg % EtOH at RT	JLV101 = 20.2 nmol/L (5.8 ng/mL)	JLV107 = 7.2 nmol/L (2.1 ng/mL)	3.1	11.2 nmol/L (3.2 ng/mL)	45
250 mL buffer + 350 mg % EtOH	JLV101 = 20.2 nmol/L (5.8 ng/mL)	JLV105 = 6.5 nmol/L (1.9 ng/mL)	3.1	10.1 nmol/L (2.9 ng/mL)	50
Tube dialysis	JLV101 = 20.2 nmol/L (5.8 ng/mL)	JLV106 = 12.3 nmol/L (3.5 ng/mL)	1	-	40
Tube dialysis at 37°C	JLV118 = 20.1 nmol/L (5.8 ng/mL)	(+) JLV157 = 4.1 nmol/L (1.2 ng/mL)	1		80
		JLV 158 = 4.0 nmol/L (1.2 ng/mL)	1		80

2.3.4 Ultrafiltration results

As mentioned in the introduction of this chapter, ultrafiltration is a similar method to equilibrium dialysis, and it was used as a confirmatory method to our research.

The percentage of displacement is minimal, possibly representing fT in serum thus showing that EtOH does not displace T from its binding proteins.

The results can be found in Table 2.9 and Appendix 8.13.

Table 2.9 Summary from serum samples using ultrafiltration

IA (control)	Experiment type	IA (retentate)	IA (filtrate)	% of displacement (filtrate & control)
JLV121 = 10.3 nmol/L (2.9 ng/mL)	Control sample	JLV149 = 9.7 nmol/L (2.8 ng/mL)	JLV150 = 0.05 nmol/L (0.01 ng/mL)	0.5
JLV121 = 10.3 nmol/L (2.9 ng/mL)	Sample + 350 mg %	JLV151 = 10.9 nmol/L (3.1 ng/mL)	JLV152 = 0.04 nmol/L (0.01 ng/mL)	0.4
JLV121 = 10.3 nmol/L (2.9 ng/mL)	Sample + 700 mg %	JLV153 = 10.5 nmol/L (3.0 ng/mL)	JLV154 = 0.05 nmol/L (0.01 ng/mL)	0.5
JLV121 = 10.3 nmol/L (2.9 ng/mL)	Sample + 1400 mg %	JLV155 = 10.2 nmol/L (2.9 ng/mL)	JLV156 = 0.03 nmol/L (0.01 ng/mL)	0.3

2.3.5 Conclusions

The work developed was to verify if an acute intoxication of EtOH, performed *in vitro*, was enough to displace T from its binding proteins in order to increase the concentration of circulating fT.

Although dissociation was verified through equilibrium dialysis, it was not enough to prove this hypothesis. In the experiments at room temperature, and with variations in the amounts of EtOH, there was a dissociation of approximately 50 %, which corresponds to the fraction released from albumin that would naturally occur through diffusion after that period of time due to the weaker binding affinity T has with that protein.

In the cases where the percentage of displacement was high (37 °C experiments), there was however no significant difference between the positive control and the sample to which EtOH had been added.

The fact that T is not displaced from its binding proteins due to alcohol was also supported with the ultrafiltration experiments, where no displacement was observed.

The hypothesis that, alcohol in serum could reach a concentration sufficient to displace T from its binding proteins such that T is more rapidly cleared than normal, and thereby increase T in urine and hence T/E ratio, was therefore disproved.

In the next chapter, we will verify if two different doses of alcohol ingested within an hour will influence urinary T/E in females and males, with all the samples being analysed by GC-MS.

Chapter 3 VALIDATION AND SAMPLE ANALYSIS OF A GC – MS METHOD FOR URINARY T, E AND 5α-DHT

3.1 Introduction

This chapter will describe the effect of rapid drinking of alcohol on the urinary T/E in women and men.

In this chapter urinary T/E, based on peak height ratios was calculated, and the concentrations of T, E and 5α−DHT (a product of T biosynthesis) in urine determined after 4 and 8 units of alcohol ingestion in 1 h, in females and in males. For that, a sufficiently sensitive validated GC-MS method was employed. This analytical technique has already been described in detail in Chapter 2.

In the UK 1 unit of alcohol is the equivalent to 10 mL. In the case of spirits which are 40 % alcohol by volume (ABV), 1 unit corresponds to 25 mL.

Exogenous testosterone is the most common finding in anti-doping laboratories. The urinary excretion of T and E is approximately equal as shown by Donike *et al.* with 50 healthy men (non-steroid users) having a T/E of 1.13 ± 0.57 SD (range 0.12-4.44) and 47 normal females having a T/E at 1.29 ± 0.89 SD (range 0.26-2.90) (Donike et al., 1983); and also reported by Van Renterghem *et al.* in an athlete population, 2,027 male samples showing a mean value for T/E of 1.64 (0.95-2.06 inter-quartile range, and 1,004 female samples having a mean of 1.14 (0.72-1.38) (Van Renterghem et al., 2010)). The inter-quartile ranges were calculated as the data did not follow a Gaussian distribution.

The method employed in the Drug Control Centre for the measurement of T/E in urine incorporates solid phase extraction (SPE), enzyme hydrolysis, liquid-liquid extraction and derivatisation by trimethylsilylation (Donike and Zimmermann, 1980, Kicman, 2010) followed by GC-MS with measurement of peak heights as an initial estimate.

3.1.1 Choice of calibrants

The chromatographic method chosen (Kicman et al., 1995) enabled the good separation of the steroids to be analysed.

The upper population range for urinary testosterone and epitestosterone concentrations has been previously reported (Ayotte et al., 1996, Geyer et al., 1996) and may be found in

Table 3.1.

Table 3.1 Upper population reference ranges (97.5th percentile) for endogenous steroids in ng/mL

Steroid	97.5th percentile	
	Females	Males
Testosterone	60	140
Epitestosterone	40	110

Since our purpose was to investigate increases in urinary T/E, and subsequent changes in T, E and 5α-DHT urinary concentrations, the 97.5th percentile from the published work by Van Renterghem *et al.* will be presented in the table below (

Table 3.2) and discussed in section 3.3 (Van Renterghem et al., 2010). The authors analysed samples from Caucasian males (n=2,027) and Caucasian females (n=1,004). Quartile 1 of the population is represented by Q1, and quartile 3 by Q3.

Table 3.2 Comparison between females and males in monitored relevant steroids in a sports population

Steroid	Gender	Q1	Mean	Q3	97.5th percentile
Urinary T/E	Females	0.72	1.14	1.38	2.65
	Males	0.95	1.64	2.06	4.33
Testosterone	Females	7	12	14	29.4
	Males	21	29	35	103
Epitestosterone	Females	7	13	17	28.5
	Males	15	29	35	88.9
5α-DHT	Females	6	9	12	20.5
	Males	5	7	9	21.5

A check of the calibration range required was made by analysis of some 33 routine samples (Table 3.3).

Table 3.3 Estimated concentration in routine samples (ng/mL)

Steroid	Lowest concentration	Mean concentration	Highest concentration
Testosterone	4	40	211
Epitestosterone	3	31	103
5α-DHT	2	15	35

Men and women excrete androgens, predominately as conjugated steroids, at different concentrations. Eleven calibrants were, therefore, chosen that would cover both ranges. Five calibrants covered a low range (females) and five a high range (males) with a sixth calibrant as an overlapping point (Table 3.4).

Table 3.4 Calibrants selected (ng/mL)

Steroid	1	2	3	4	5	6	7	8	9	10	11
Testosterone	1	2	5	10	15	25	50	75	100	125	160
Epitestosterone	1	2	5	10	15	25	50	75	100	125	160
5α-DHT	1	2	4	8	12	16	20	25	30	35	40

3.1.2 Method validation

The method was validated against accepted criteria by the International Conference of Harmonisation (CPMP/ICH/281 & 381 guidelines) for linearity, specificity, lower limit of quantification (LLOQ), accuracy and precision.

Linearity is determined using a standard curve with a minimum of six points that will define the assay range between the LLOQ and the higher limit of quantification (HLOQ). Its linear regression should have a correlation coefficient (r^2) > 0.99 and the accuracy of the mean of any calibration point should be < 15 % at any calibration point, and < 20 % at the LLOQ.

Specificity was assessed by testing at least 6 different batches of control biological matrix for the presence of chromatographic interferants, which could affect the assay performance.

The LLOQ is the smallest concentration of analyte that the instrument can measure for which the S/N is 1:10, and where the concentrations replicates in sextuplicates have a CV < 20 %.

Accuracy is the difference between the measured concentration and the nominal one. This needs to be ± 15% of nominal concentration and is determined both within assay and between assays.

Precision is the level of agreement between a set of measurements and needs to be < 10 %. It is measured in a single assay (repeatability) and also between assays (reproducibility).

3.1.3 Study design

The units of alcohol administered to our volunteers had to be enough to provoke inebriation but below toxicity levels.

Assuming normal body composition, for a male weighing 70 kilograms (kg) drinking 4 units of alcohol, the blood alcohol concentration would be 0.45 g/kg of body weight (according to the Widmark Factor), or 67 mg/100 mL of blood, which is below the drink driving limit (80 mg/100 mL of blood). For 8 units the blood alcohol concentration would be 0.9 g/kg of body weight or 134 mg/100 mL, which is 1.7 times the drink driving limit.

For a female, assuming normal body composition, and a weight of 60 kg, the blood alcohol concentration for 4 units would be 0.53 g/kg of body weight, or 96 mg/100 mL of blood which is 1.2 times the drink driving limit. For 8 units, the results would be 1.06 g/kg of body weight or 192 mg/100 mL of blood, which is 2.4 times the drink driving limit.

3.2 Material and methods

3.2.1 Materials

T, E, 5α-DHT were obtained from Promochem (Teddington, UK). Potassium carbonate, sodium sulphate, potassium dihydrogen orthophosphate, anyhydrous disodium hydrogen orthophospate, sodium chloride, sodium dihydrogen phosphate dehydrate, sodium hydroxide, hydrochloric acid, diethyl ether and methanol (HPLC grade) were all obtained from Fisher Scientific (Loughborough, UK). Ammonium iodide (both analytical grade) were obtained from BDH Chemicals (Leicestershire, UK). MSTFA was obtained from Sigma-Aldrich (Germany); and ethanethiol, urea, bovine albumin and creatinine from Sigma-Aldrich (Poole, UK). The deuterated internal standards d_3T, d_3E and $d_3$5α-DHT were supplied by the National Analytical Reference Laboratory (Sidney, Australia). Escherichia coli β-glucuronidase (K-12) was obtained from Roche (Switzerland). Vacutainers™ and all the relevant blood paraphernalia were purchased from BD Diagnostics (Oxford, UK).

3.2.2 Preparation of screen and calibrant standards

3.2.2.1 Phosphate buffer 0.1 M, pH 6.2

Potassium dihydrogen orthophosphate (11.3 g) and anhydrous disodium hydrogen orthophosphate (2.4 g) were added to 900 mL of purified water in a 1 L volumetric flask, and sonicated for 10 min. It was then made up to 1 L by adding purified water and transferred into a screw top container. The pH was checked (pH 6.2 ±0.1) and, if necessary, adjusted with glacial acetic acid (17.47 M) or sodium hydroxide solution (10 M), and stored in the fridge.

3.2.2.2 *Eschericia coli* enzyme solution – hydrolysis solution

To the 0.1 M phosphate buffer solution (3.2.2.1) 2 mL of *E.coli* β-glucuronidase was added and then stored at +4ºC until needed. The acitivity of the enzyme as describe in its technical information sheet, is of 140 U/mg at 37 °C. This is defined when using 4-nitrophenyl-β-D-glucuronide as substrate (Roche Applied Science, 2012).

3.2.2.3 Combined internal standard mixture

To prepare the stock solution the ampoules containing the internal standard of interest (d_3T, d_3E and $d_3$5α-DHT was broken and its contents (approximately 1 mg) dissolved in methanol and transferred into a 10 mL volumetric flask. The ampoule was washed with methanol approximately 10 times. The solutions were kept in the freezer at an indicated temperature -20 °C. The final concentration of each methanolic solution may be found in Appendix 8.14. To prepare the stock solution a dilution was made (Appendix 8.15).

The volume corresponding to each deuterated steroid was approximately measured with a volumetric syringe and added to a 100 mL volumetric flask and made up in methanol. The solution was then transferred to a 200 mL bottle and 100 mL of purified water added to give the final internal standard working solution at concentrations of d_3T and d_3E at 25 ng/mL, and 16 ng/mL of $d_3$5α-DHT.

3.2.2.4 Derivatising reagent

Ammonium iodide (100 mg) was weighed into a 20 mL round bottomed centrifuge tube and covered in foil. To this, MSTFA (5 mL) was added, and heated to 100 °C until the ammonium iodide had dissolved. After cooling, ethanethiol (300 μL) was added and the tube flushed with nitrogen, sealed and mixed thoroughly. This gave a final stock solution of MSTFA:ammonium iodide:ethanethiol 100:2:6.

The working solution was prepared by diluting the stock solution (4 mL) into a new bottle of MSTFA (25 g, 23.25 mL). This solution had a concentration of MSTFA:ammonium iodide:ethanethiol 1000:3:9. This was capped, mixed thoroughly and flushed with nitrogen before resealing. The mixture was then split into 2 mL amber glass vials, previously flushed with nitrogen, capped and kept in the fumehood.

3.2.2.5 Synthetic urine

The preparation of synthetic urine available in our laboratory was followed. Urea (36.5 g) was added to 1.5 L of purified water, and mixed until the crystals dissolved. Sodium chloride (15 g),

potassium chloride (9 g), sodium didhydrogen phosphate dihydrate (12.8 g) was also added to the solution and mixed until the solution was clear. Creatinine (4 g) and bovine serum albumin (100 mg) were added, the solution was thoroughly mixed and the pH adjusted to between 5 - 7 with either hydrochloric acid 1 M, or sodium hydroxide 1 M. The specific gravity (SG) was measured and if it was higher than 1.020 ±0.002 the solution was diluted with purified water.

3.2.2.6 Preparation of calibrants

A stock solution of 1 mg/mL in methanol was prepared for all the compounds. Sub-stock solutions were prepared in synthetic urine at a concentration of 100 μg/mL. A second sub-stock dilution was made from the 100 μg/mL resulting in a working solution of 1 μg/mL. The calibrants final concentration can be found in Table 3.4.

3.2.2.7 Choice of quality controls

Urine was collected from 17 members of the laboratory (2 females and 15 males) to be analysed for approximate steroid concentrations. Urine collections were made in Nalgene™ polypropylene bottles.

3.2.3 Volunteer study and sample collection

Ethical approval was sought and given by the National Research Ethics Committee in the United Kingdom, for the recruitment of 20 volunteers (10 females and 10 males) from whom we collected blood and urine after alcohol ingestion.

The samples were labelled with an alphanumeric code, e.g. TαF-4U-βδ, where α is the number of the volunteer, β the letter indicating the type of sample (U for urine and S for serum), and δ the time of collection.

The volunteers were aged between 18-35 years old (reflecting the typical age range of adult athletes) and were required to ingest 4 and 8 units of alcohol on two different occasions with a minimum of a two-week interval. The alcohol was in the form of vodka (40 %) – 1 unit equals to

8 grams of alcohol which equals 1 shot of 25 mL of vodka (40 % ABV), therefore 4 units of vodka would be slightly under half a glassful and 8 units slightly under a glassful – diluted in 300 mL of tonic water or orange juice.

On the screening day, which took place 1 week before the study, a physician assessed the volunteer's health and collected a blood sample (10 mL). The sample was sent for analysis of liver enzymes and blood coagulation factors.

The volunteer also filled in a questionnaire providing information regarding their weight, height, ethnic origin (40 % of Asian population has a gene deletion and lacks the enzyme to metabolize acetaldehyde which is one of alcohol's metabolites and were therefore excluded from this study), if they were pregnant or using oral contraceptive, and which one (for females only), if they were taking any liver medication, if they had kidney failure, what were their drinking habits (how many units per week) and when was the last time they had drunk alcohol. The volunteers were asked to be alcohol-free for 3 days prior to sample donation, which is roughly the amount of time it takes until alcohol metabolites are not longer detectable.

For the experiment day, the volunteers were asked once again to be alcohol-free for 3 days.

The administration of alcohol to the subjects (5 people per group) was performed at lunch time (12 a.m.) accompanied by a light lunch (sandwiches), in the study suite at King's College London, Waterloo campus. The participants were asked to have a light breakfast (non-cooked) on the morning of the study.

The volunteers were cannulated by the physician at the beginning of the experiment prior to alcohol consumption for up to 6 h.

The volunteers were asked to ingest their alcoholic drink over a 1 h period and were asked to drink 125 mL of water every hour for the first 7 hours post completion of administration to ensure the same water intake in all the participants.

A 20 mL blood sample was taken prior to alcohol ingestion and hourly until 6 h, then singularly at 24 h and 48 h after alcohol intake.

Urine samples were collected prior to administration and every hour until 7 hour, then at 10 h, 24 h, 48 h and 72 h. Urine and serum samples were stored at -20 °C prior to analysis.

A breath test was performed before the volunteers were allowed to leave the facilities, to ensure that their breath alcohol concentration was below the drink driving limit (35 μg/dL).

Serum was analysed by liquid-chromatography tandem mass-spectrometry (LC-MS/MS) to measure T, E, its glucuronides and sulfates, androstenedione, ethyl glucuronide and ethyl sulphate. Immunoassay was used to measure LH.

The urine samples were analysed by GC-MS to measure the T/E ratio, concentration of T, E and 5α-DHT, and by LC-MS/MS to measure EtG and EtS.

Alcohol measurements in serum were performed by Mr. Peter Streete from the Medical Toxicology Laboratory, GSTS Pathology, in St. Thomas' Hospital.

All the calibrants and samples were plotted using a linear weighted regression calibration using MultiCalc® 2000 software program from PerkinElmer®.

The statistical treatment was performed using the software Statistical Package for Social Sciences (SPSS®), version 18, from SPSS® Inc, Chicago, IL, USA. The results were statistically treated by using a general linear model with repeated measures and the significance limit was set to p-value <0.05. This statistical treatment tests for any changes, and whether or not they are statistically significant. From the statistical graphs and tables it is then possible to verify if any significant changes are an increase or a decrease.

3.2.4 Analysis of samples

3.2.4.1 Extraction procedure

For the extraction, 2 mL of each sample was pippeted into a polypropylene tubes along with 100 μL of internal standard, mixed in a vortex mixer and centrifuged at 140 *g* for 5 min. Isolute C8 cartridges were conditioned with methanol (3 mL) and equilibrated with water (3 mL). Each

sample was loaded onto the cartridge and washed with water (3 mL). The sample was then eluted with methanol (3 mL) and dried at 60° C ±5 °C degrees under nitrogen gas.

For the hydrolysis, 1 mL of *E.coli* solution was added to each tube and incubated for 1 h at 50° C ±5 °C degrees.

After the incubation, potassium carbonate was added to each sample (100 mg) followed by 5 mL of diethyl ether prior to vortex mixing for 30 seconds, with the organic layer subsequently removed and placed into a 20 mL glass tube. Anhydrous sodium sulphate was then added whilst vortexing and the sample allowed to stand for 2 min. The samples were centrifuged at 700 rpm (100 *g*) for 5 min before decanting off the organic phase into a 10 mL glass tube and rinsed with 2 mL of diethyl ether, which was then pooled with the previous fraction. The solvent was evaporated to dryness at 30 °C ±5 °C under nitrogen gas and put into a desiccator containing sodium hydroxide pellets and phosphorous pentoxide for at least 30 min.

The test tubes were then flushed with nitrogen and 20 μL of silylating reagent (MSTFA:NH_4I:ethanethiol 1000:3:9) was immediately added. Samples were heated at 60 °C ±5 °C for 20 min, and then allowed to cool. Finally 20 μL of dodecane was added and the samples vortexed before transfering into an autosampler vial and analysed by GC-MS.

3.2.4.2 GC-MS conditions

The GC column was HP-1 (methylsiloxane) (25 m, 0.2 mm, 0.11 μm) from Agilent. An initial oven temperature of 180 °C was held for 1 min, ramping at 2 °C/min until it reached 228 °C, and then 35 °C/min to 300°C and held for 2.50 min. The total run time was 29.56 min.

The injection volume was 1 μL in splitless mode.

3.3 Results and discussion

3.3.1 Method validation

The LLOQ was measured using the calibrant standards prepared in synthetic urine, and the lowest calibrant was prepared at the LLOQ, where S/R was 10:1. For T, E and 5α-DHT this was 1 ng/mL.

Preparing the calibrant standards in synthetic urine was the method of choice due to it being steroid free. To test the suitability of this synthetic matrix a comparison was made between a urine sample containing approximately 5 ng/mL of T and 6 replicates were extracted, together with 6 replicates of a calibrant standard at the same concentration. A two sample F-test variance was performed ($p > 0.05$) indicative that the variances are not significantly different at least at 5 ng/mL, therefore the synthetic urine was used as a matrix for the calibration standards.

The quality controls were prepared in urine in sufficient amount to last for the 3-day validation and for the sample analysis to ensure that no error in precision came from the original QC solution. Since two ranges of calibrants were being used, two QCs for each range were chosen. The low QC was closer to the lower point of the respective range, and the high QC closer to the highest calibrant. The QCs were labelled LOW1 QC and HIGH1 QC for the lowest range, and LOW2 QC and HIGH2 QC for the highest range.

From the 17 urine samples collected, only 2 females and 2 males had the urinary concentrations of the steroids of interest in the desired range and they were asked to provide approximately 1 L of urine during 1 day to prevent differences in steroid concentration. The preliminary concentration of each steroid of interest calculated was done again by using a peak height ratio with the internal standard. The concentrations can be found in Table 3.5.

Table 3.5 Preliminary check for QC urinary concentrations (ng/mL)

Steroid	Urine A	Urine B	Urine C	Urine D
Testosterone	0.33	3.0	35.2	53.3
Epitestosterone	2.62	6.5	32.7	32.1
5α-DHT	12.8	15.0	38.1	36.8

The ideal concentration for a low QC would be for it to be within 25 % of the lower end of the calibrant range, and for the high QC to be between 75-100 % of the highest concentration in the calibrant range. An appropriate concentration for the QCs was thus chosen, and urines A, B and D spiked with methanolic solutions (no more than 1 % of total volume) with the steroids of interest. The prepared concentrations may be found in Table 3.6.

Table 3.6 Expected concentration for each QC (ng/mL)

Steroid	LOW1	HIGH1	LOW2	HIGH 2
Testosterone	3	20	32	127
Epitestosterone	3	21	31	130
5α-DHT	12	15	22	35

Validation was performed by extracting each QC six times on three different days. The concentration of each QC was calculated by plotting it against a calibration curve with standards prepared fresh on the day. The parameters used were:

Equation 3.1 Accuracy (within assay)

$$\frac{\qquad\qquad}{\qquad\qquad}$$

(< ± 20 % LLOQ, < ± 15 % other points)

Equation 3.2 Average accuracy (between assay)

(< ± 10 %)

Equation 3.3 Repeatability

(< 10 %)

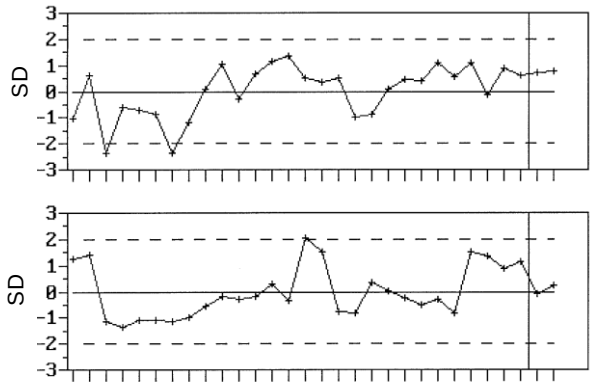
Equation 3.4 Reproducibility

(< 10 %)

Equation 3.5 Average precision (between assay)

(< 10 %)

The QCs were monitored in every run and they were within the acceptance criteria (±2 SD).



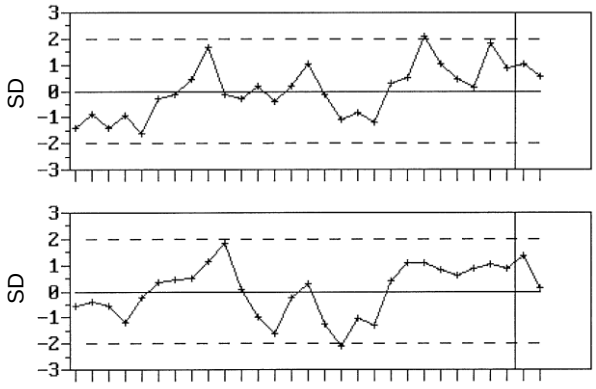
T LOW QC (low range)

Mean concentration = 3.8 ng/mL (± 0.3)

T HIGH QC (low range)

Mean concentration = 23.1 ng/mL (± 1.6)

Figure 3.1 Low and High QC for testosterone in the low range



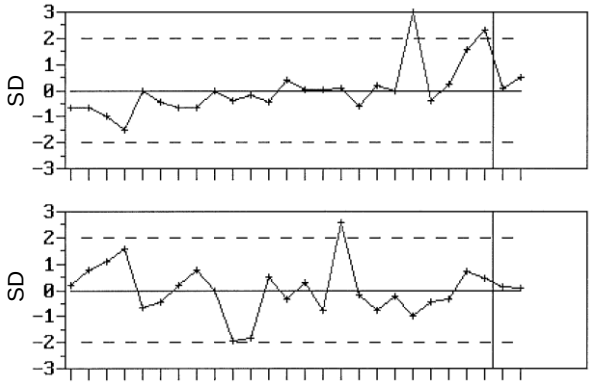
E LOW QC (low range)

Mean concentration = 3.9 ng/mL (± 0.3)

E HIGH QC (low range)

Mean concentration = 22.9 ng/mL (± 1.9)

Figure 3.2 Low and High QC for epitestosterone in the low range



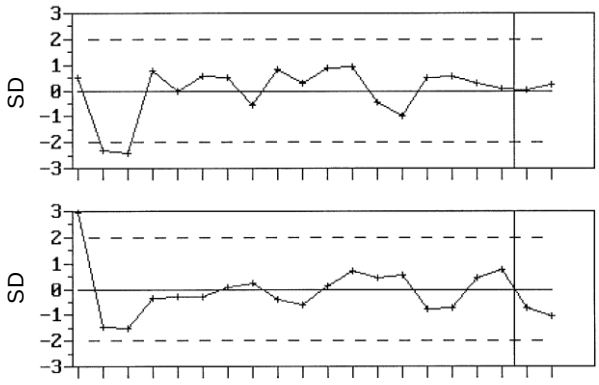
5α-DHT LOW QC (low range)

Mean concentration = 4.0 ng/mL (± 0.3)

5α-DHT HIGH QC (low range)

Mean concentration = 13.8 ng/mL (± 0.6)

Figure 3.3 Low and High QC for 5α-DHT in the low range



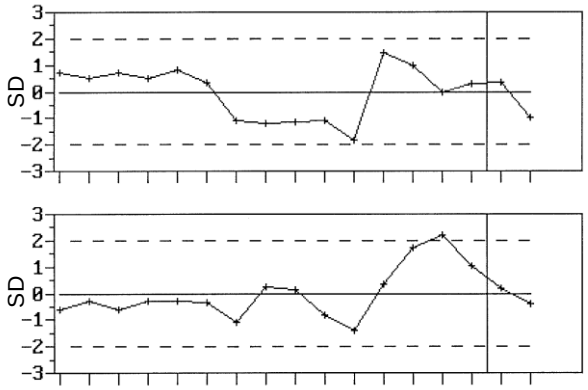
T LOW QC (high range)

Mean concentration = 32.8 ng/mL (± 3.5)

T HIGH QC (high range)

Mean concentration = 114.9 ng/mL (± 14.8)

Figure 3.4 Low and High QC for testosterone in the high range



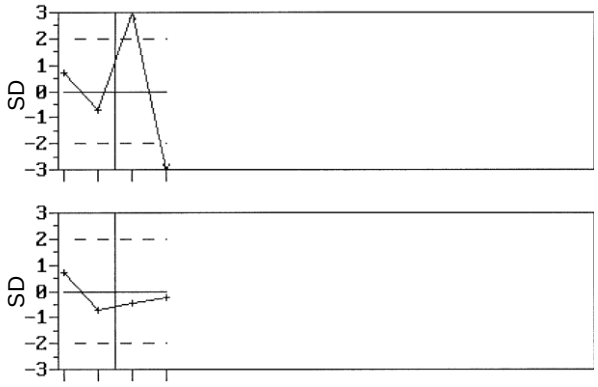
E LOW QC (high range)

Mean concentration = 36.7 ng/mL (± 4.3)

E HIGH QC (high range)

Mean concentration = 100.1 ng/mL (± 8.4)

Figure 3.5 Low and High QC for epitestosterone in the high range



5α-DHT QC (high range)

Mean concentration = 21.6 ng/mL (± 0.14)

5α-DHT HIGH QC (high range)

Mean concentration = 31.5 ng/mL (± 3.3)

Figure 3.6 Low and High QC for 5α-DHT in the high range

3.3.2 Method validation for the quantification of urinary T

3.3.2.1 Lower limit of quantification

The LLOQ, as mentioned previously, is defined by a S/N of 1:10 between the analyte's peak and the adjacent peak. The LLOQ for T was 1 ng/mL (Figure 3.7).

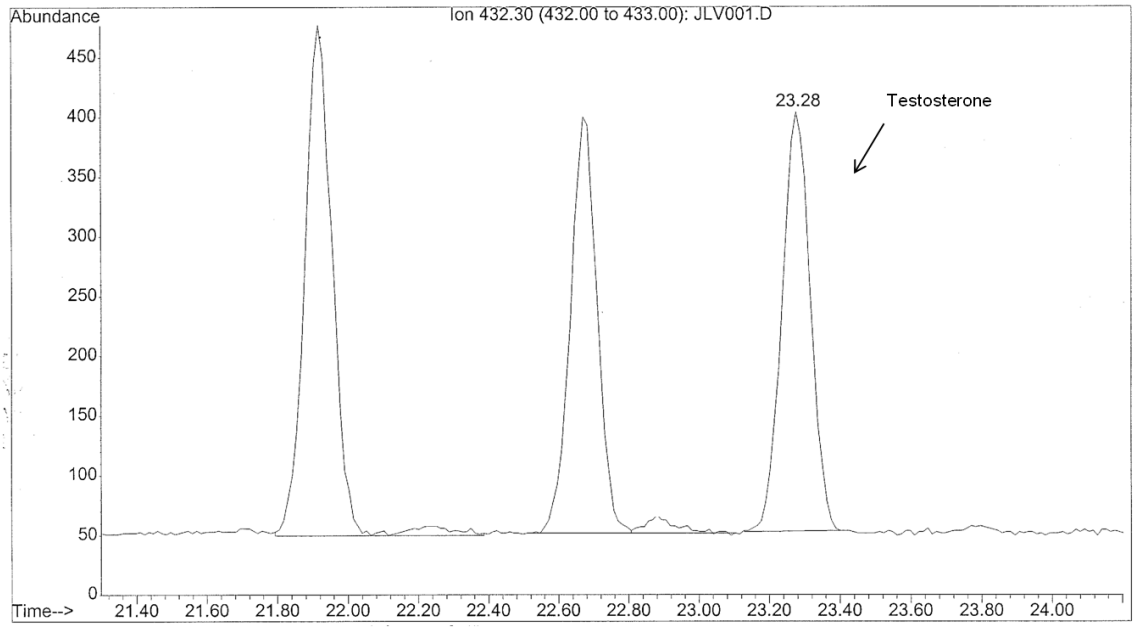


Figure 3.7 Typical chromatogram obtained at a lower limit of quantification for T (1 ng/mL)

3.3.2.2 Calibration range

The lowest calibrant, at the LLOQ, had a percentage accuracy (within assay) <±20%, and the remaining calibrants <±15 %, passing the validation criteria. The linearity of the calibration plots (r^2 value), for the 3 day validation of T, from 1 to 25 ng/mL was 0.9987, 0.9995 and 0.9977; and from 25 to 160 ng/mL were 0.9900, 0.9963 and 0.9980, respectively.

3.3.2.3 Quality controls validation

Table 3.7 Validation of urinary T for LOW1 and HIGH1 QCs (low range)

	Day 1	Day 2	Day 3
LOW1 QC (ng/mL)	3.1	3.6	3.5
	3.6	3.3	5.0
	3.3	3.4	3.5
	3.4	3.3	3.9
	3.1	3.7	3.6
	3.4	3.9	3.9
Mean (ng/mL)	3.3	3.5	3.9
% Accuracy	-10.3	-4.4	6.1
SD (ng/mL)	0.2	0.3	0.6
% CV	5.7	7.1	14.6

	Day 1	Day 2	Day 3
HIGH1 QC (ng/mL)	21.3	21.3	25.2
	20.6	22.5	23.4
	16.8	21.8	25.8
	20.0	23.6	21.8
	21.5	22.9	24.4
	20.9	22.7	24.4
Mean (ng/mL)	20.2	22.5	24.2
% Accuracy	-4.0	6.9	15.1
SD (ng/mL)	1.8	0.8	1.4
% CV	8.7	3.6	5.9

Table 3.8 Validation parameters for urinary T LOW1 QC and HIGH1 QC

LOW1 QC	
%Accuracy (between assay)	-2.9
%Precision (within assay)	9.1
%Precision (between assay)	9.4

HIGH1 QC	
%Accuracy (between assay)	7.3
%Precision (within assay)	3.1
%Precision (between assay)	5.9

Table 3.9 Validation of urinary T for LOW2 and HIGH2 QCs (high range)

	Day 1	Day 2	Day 3
LOW2 QC (ng/mL)	24.7	31.9	35.9
	27.6	29.8	33.0
	24.8	29.8	33.4
	27.9	32.0	31.7
	25.3	31.7	30.7
	27.2	32.4	30.9
Mean (ng/mL)	26.2	31.3	32.6
% Accuracy	-15.4	0.8	5.1
SD (ng/mL)	1.5	1.2	2.0
% CV	5.6	3.7	6.0

	Day 1	Day 2	Day 3
HIGH2 QC (ng/mL)	93.0	109.3	101.0
	92.1	105.9	114.6
	99.8	103.8	117.6
	99.6	105.7	103.2
	99.5	108.0	111.9
	100.9	105.1	102.9
Mean (ng/mL)	97.5	106.3	108.5
% Accuracy	-12.2	-4.2	-2.2
SD (ng/mL)	3.9	2.0	7.0
% CV	4.0	1.9	6.5

Table 3.10 Validation parameters of urinary T for LOW2 QC and HIGH2 QC

LOW2 QC	
%Accuracy (between assay)	-3.2
%Precision (within assay)	5.1
%Precision (between assay)	5.1

HIGH2 QC	
%Accuracy (between assay)	-6.2
%Precision (within assay)	4.1
%Precision (between assay)	4.1

The QCs in the low range and in the high range passed the criteria so the method was successfully validated for T.

3.3.3 Method validation for the quantification of urinary E

3.3.3.1 Lower limit of quantification

The LLOQ for E was 1 ng/mL, lowest calibrant, and can be found in Figure 3.8.

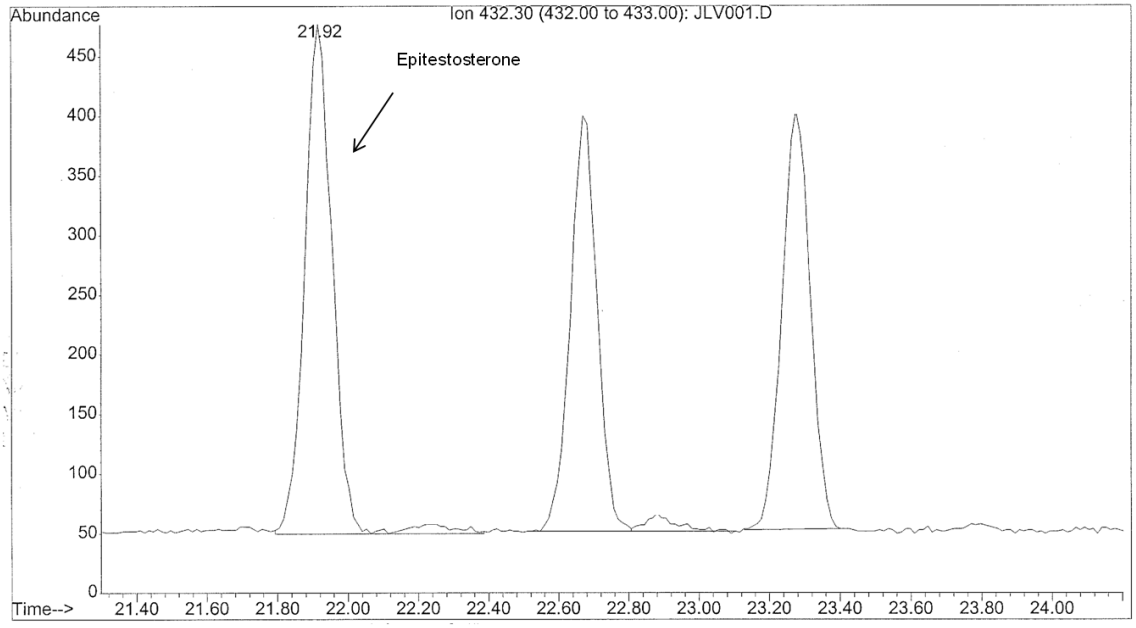


Figure 3.8 Typical chromatogram obtained at a lower limit of quantification for E at 1 ng/mL

3.3.3.2 Calibration range

All calibrants were accurate <±15 %, and <±20 % at the LLOQ. The low range (1-25 ng/mL) was linear (r^2 value) throughout the 3 days with values of 0.9938, 0.9994 and 0.9995. The high range's r^2 values were 0.9906, 0.9966 and 0.9956, for a concentration range of 25-160 ng/mL.

3.3.3.3 Quality control validation

Table 3.11 Validation of urinary E for LOW1 and HIGH1 QCs (low range)

	Day 1	Day 2	Day 3
LOW1 QC (ng/mL)	3.6	3.7	3.9
	4.0	3.8	5.3
	3.8	3.5	4.1
	3.4	3.6	3.9
	3.4	3.5	4.2
	3.3	3.9	3.7
Mean (ng/mL)	3.6	3.6	4.2
% Accuracy	-3.5	-1.4	13.0
SD (ng/mL)	0.3	0.2	0.6
% CV	8.0	4.6	13.4

	Day 1	Day 2	Day 3
HIGH1 QC (ng/mL)	21.9	20.7	23.5
	21.0	21.2	22.6
	18.0	20.1	25.6
	20.5	21.0	21.0
	20.4	21.3	24.0
	20.5	21.3	25.1
Mean (ng/mL)	22.3	20.9	23.6
% Accuracy	1.3	-4.8	7.4
SD (ng/mL)	1.3	0.5	1.7
% CV	5.9	2.2	7.1

Table 3.12 Validation parameters of urinary E for LOW1 QC and HIGH1 QC (low range)

LOW2 QC	
%Accuracy (between assay)	2.7
%Precision (within assay)	8.7
%Precision (between assay)	8.9

HIGH2 QC	
%Accuracy (between assay)	1.3
%Precision (within assay)	5.1
%Precision (between assay)	5.2

Table 3.13 Validation of urinary E for LOW2 and HIGH2 QCs (high range)

	Day 1	Day 2	Day 3
LOW2 QC (ng/mL)	27.0	30.5	39.8
	29.1	31.6	37.3
	26.3	30.1	35.8
	26.6	29.8	34.8
	31.0	30.7	34.8
	28.8	29.7	34.8
Mean (ng/mL)	28.1	30.4	36.2
% Accuracy	-14.7	-7.9	9.7
SD (ng/mL)	1.8	0.7	2.0
% CV	6.4	2.3	5.5

	Day 1	Day 2	Day 3
HIGH 2QC (ng/mL)	88.6	96.2	95.1
	98.8	93.2	109.7
	98.9	92.9	105.4
	95.6	93.1	105.6
	93.1	95.0	104.6
	99.1	88.9	100.2
Mean (ng/mL)	95.7	93.2	103.4
% Accuracy	-2.4	-4.9	5.5
SD (ng/mL)	4.2	2.5	5.1
% CV	4.4	2.6	4.9

Table 3.14 Validation parameters of urinary E for LOW2 QC and HIGH2 QC (high range)

LOW2 QC	
%Accuracy (between assay)	-4.3
%Precision (within assay)	4.8
%Precision (between assay)	4.8

HIGH2 QC	
%Accuracy (between assay)	-0.6
%Precision (within assay)	4.0
%Precision (between assay)	4.0

In both ranges, the QCs passed the validation criteria.

3.3.4 Method validation for the quantification of urinary 5α-DHT

3.3.4.1 Lower limit of quantification

The LLOQ for 5α-DHT was 1 ng/mL, (S/N 1:10) as shown in Figure 3.9.

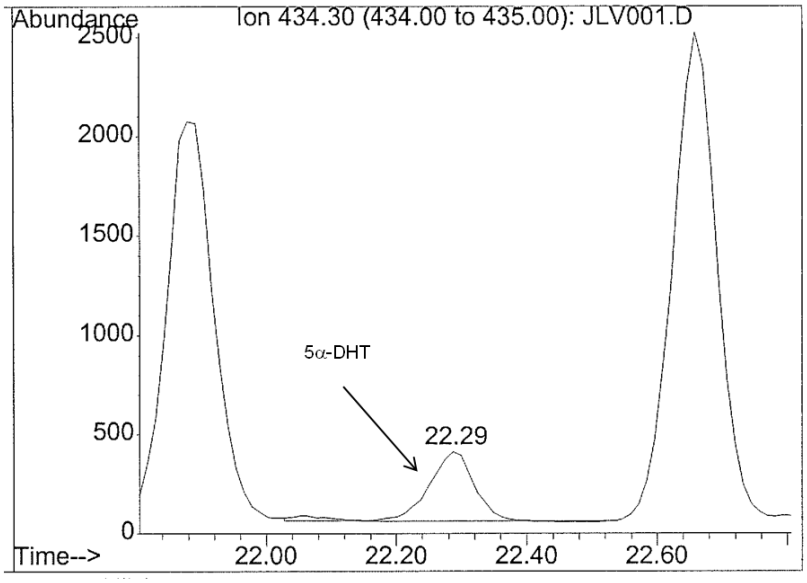


Figure 3.9 Typical chromatogram obtained at a lower limit of quantification for 5α-DHT at 1 ng/mL

3.3.4.2 Calibration range

Calibrants for 5α-DHT were accurate <±15 %, and <±20 % at the LLOQ. For the concentration range 1-16 ng/mL (low range) the linearity (r^2) throughout the 3 days were 0.9983, 0.9985 and 0.9992. For the range 16-40 ng/mL (high range), the linearity values (r^2) were 0.9987, 0.9964 and 0.9928.

3.3.4.3 Quality control validation

Table 3.15 Validation of urinary 5α-DHT for LOW1 and HIGH1 QCs (low range)

	Day 1	Day 2	Day 3
LOW1 QC (ng/mL)	3.7	4.0	3.8
	4.4	4.2	4.7
	3.5	3.7	3.6
	4.0	4.1	4.1
	3.4	3.9	3.8
	3.4	4.0	3.9
Mean (ng/mL)	3.7	4.0	4.0
% Accuracy	-6.4	-1.1	-0.4
SD (ng/mL)	0.4	0.2	0.4
% CV	10.4	3.8	10.2

	Day 1	Day 2	Day 3
HIGH1 QC (ng/mL)	14.5	13.4	13.9
	13.9	13.2	12.7
	10.9	13.6	12.7
	13.0	14.4	13.7
	13.1	12.2	15.4
	13.7	13.5	15.8
Mean (ng/mL)	13.2	13.4	14.0
% Accuracy	1.4	3.1	7.9
SD (ng/mL)	1.2	0.7	1.3
% CV	9.4	5.2	9.5

Table 3.16 Validation parameters of urinary 5α-DHT for LOW1 QC and HIGH1 QC (low range)

LOW2 QC	
%Accuracy (between assay)	-2.6
%Precision (within assay)	8.1
%Precision (between assay)	8.1

HIGH2 QC	
%Accuracy (between assay)	4.1
%Precision (within assay)	8.0
%Precision (between assay)	8.1

Table 3.17 Validation of urinary of 5α-DHT for LOW2 and HIGH2 QCs (high range)

	Day 1	Day 2	Day 3
LOW2 QC (ng/mL)	20.1	21.8	22.2
	22.7	23.2	22.4
	18.2	22.3	20.2
	19.5	23.0	22.9
	19.9	21.2	21.9
	20.2	21.2	19.8
Mean (ng/mL)	20.1	22.1	21.6
% Accuracy	-8.7	0.5	-1.9
SD (ng/mL)	1.4	0.9	1.2
% CV	7.2	3.9	5.8

	Day 1	Day 2	Day 3
HIGH2 QC (ng/mL)	29.8	32.1	30.5
	29.3	29.6	32.8
	32.5	28.5	34.9
	28.3	32.6	30.8
	30.4	31.7	31.4
	29.6	31.8	29.4
Mean (ng/mL)	30.0	31.0	31.6
% Accuracy	-6.3	-3.1	-1.2
SD (ng/mL)	1.4	1.6	2.0
% CV	4.7	5.2	6.2

Table 3.18 Validation parameters for urinary 5α-DHT LOW2 QC and HIGH2 QC (high range)

LOW2 QC	
%Accuracy (between assay)	-3.4
%Precision (within assay)	5.6
%Precision (between assay)	5.6

HIGH2 QC	
%Accuracy (between assay)	-3.5
%Precision (within assay)	5.4
%Precision (between assay)	5.4

For both the low and high ranges, the QCs successfully passed the validation criteria.

3.3.5 Summary of validation criteria for T, E and 5α-DHT

All the parameters taken into account for the validation were within the established accepted criteria (Table 3.19), except for LOW2 QC for T, which had a within assay accuracy of 15.4 %. Given the fact that the preliminary concentration was performed by using a peak height ratio, some error may have come from there. The difference from the accepted criteria was only 0.4 %, and because for the other 2 days of validation the accuracy was within the criteria, this value was accepted.

Table 3.19 Summary of validation criteria

Validation parameters	Criteria
LLOQ	S/N 1:10, sextuplicates' CV % <±20 %
Linearity	r^2 value > 0.99
Accuracy (within assay)	<±20 % for LLOQ, <±15 % other points
Average accuracy (between assay)	<±10 %
Repeatability (precision in a single assay)	<±10 %
Reproducibility (precision within assay)	<±10 %
Reproducibility (precision between assay)	<±10 %

3.3.6 Sample analysis results and challenges encountered

Urinary T/E, and the concentrations of the previously validated steroids were calculated. These concentrations vary greatly between individual and depend upon the urinary density, or specific gravity (SG), of the urine sample. Only values normalized for a SG of 1.020 can be compared.

The SG of all urine samples was measured and the concentration of steroid adjusted according to the following equation:

Equation 3.6 Adjusted concentrations

In certain samples the concentration obtained was below the LLOQ (1 ng/mL). In cases where the concentration was between 0.5-1 ng/mL, a re-extraction of 4 mL was performed to verify if, by doubling the volume, the concentration would increase to the double. However, such was not the case and because we were just interested in the concentration differences rather than an absolute value, all the samples which had a concentration below the LLOQ were modified to 0.5 ng/mL for statistical treatment purposes. It was also decided to do the same in samples where the SG was below 1.004 to avoid introducing errors in highly diluted urine samples. The samples that had an SG below 1.004 were, not surprisingly, part of the group of samples with a concentration below the LLOQ.

This happened in 9 % of the samples analysed for T concentration, in 3.5 % in the samples for E concentration and in 26 % of the samples analysed for 5α-DHT concentration. The high percentage of samples below the LLOQ for 5α-DHT was expected since 5α-DHT glucuronide is excreted in very small amounts and in previous published data the undetectable percentage was in the order of 40 % (Kicman et al., 1995).

Statistical treatment was done by averaging post-administration data irrespective of gender, time or units and normalizing the data using percentage increase against pre-administration as follows:

Equation 3.7 Normalization for statistical treatment

The 72 h sample was excluded from statistical treatment since 1 male and 1 female volunteer failed to provide this sample so there was no data. The samples collected at 24 h and 48 h had

already reached baseline values and a comparison between baseline value (baseline sample taken before alcohol ingestion) and these two samples were done to ascertain if the response was different for the 4 and 8 units. The results were not statistically significant with T/E p-value =0.173, T concentration p-value=0.329, E concentration p-value=0.854 and 5α-DHT concentration p-value=0.312 therefore the last two measurements (24 and 48 h) were excluded from the statistical treatment.

As an added test of reliability for our baseline values, the mean of pre-administration sample, 24 h and 48 h was used as the baseline value.

During the study, 1 male volunteer dropped out (T3M), and it was not possible to collect blood from 1 female (T3F).

The urinary T/E for females, their T, E and 5α-DHT urinary concentrations for 4 units may be found in Appendix 8.16 and for 8 units in Appendix 8.17. For males, urinary concentrations following administration of 4 units are shown in Appendix 8.18, and for 8 units in Appendix 8.19.

The data will be presented for females first followed by the males. It will be compared against the WADA threshold and 97.5th percentile reference values in a female Caucasian athlete population (n=1,004) and in a male Caucasian athlete population (n=2,027) recently published (Van Renterghem et al., 2010).

3.3.6.1 Urinary T/E ratio

The WADA action limit for follow-up work is set at T/E < 4, and the 97.5th percentile in females is 2.65 and for males it was 4.33.

The 4 and 8 units were plotted in the same graph using a double axis, with the higher dose being below and with the y-axis on the right hand-side.

For females, the results for 4 and 8 units may be seen in Figure 3.10, and for males in Figure 3.11.

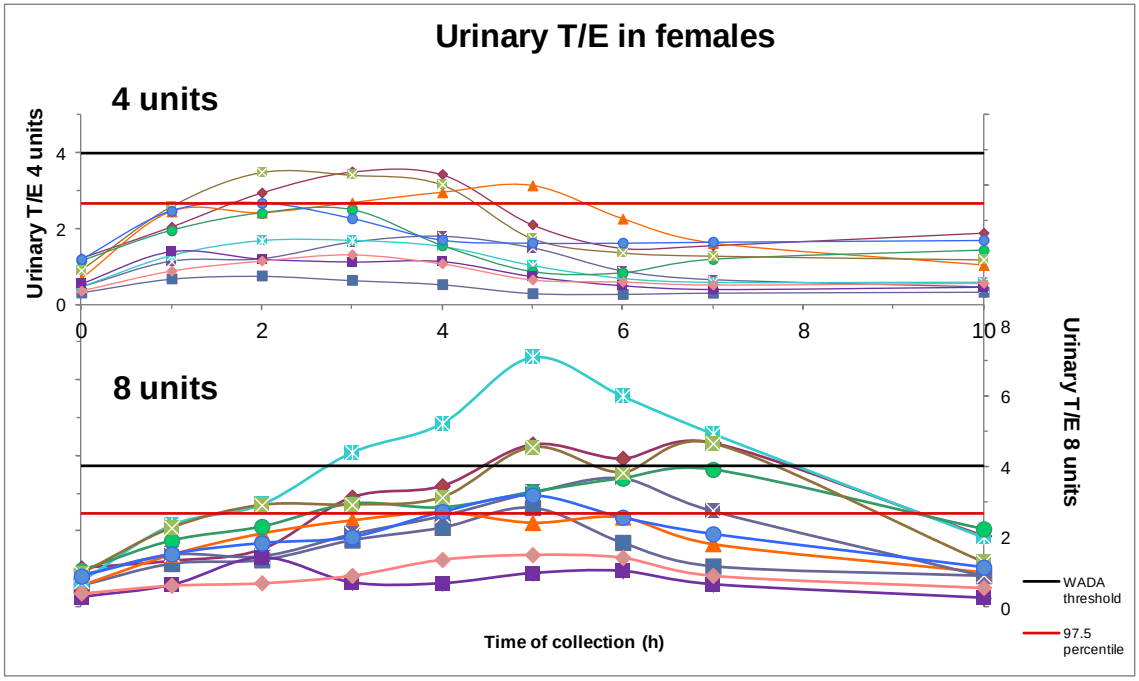


Figure 3.10 Urinary T/E in females, 4 and 8 units

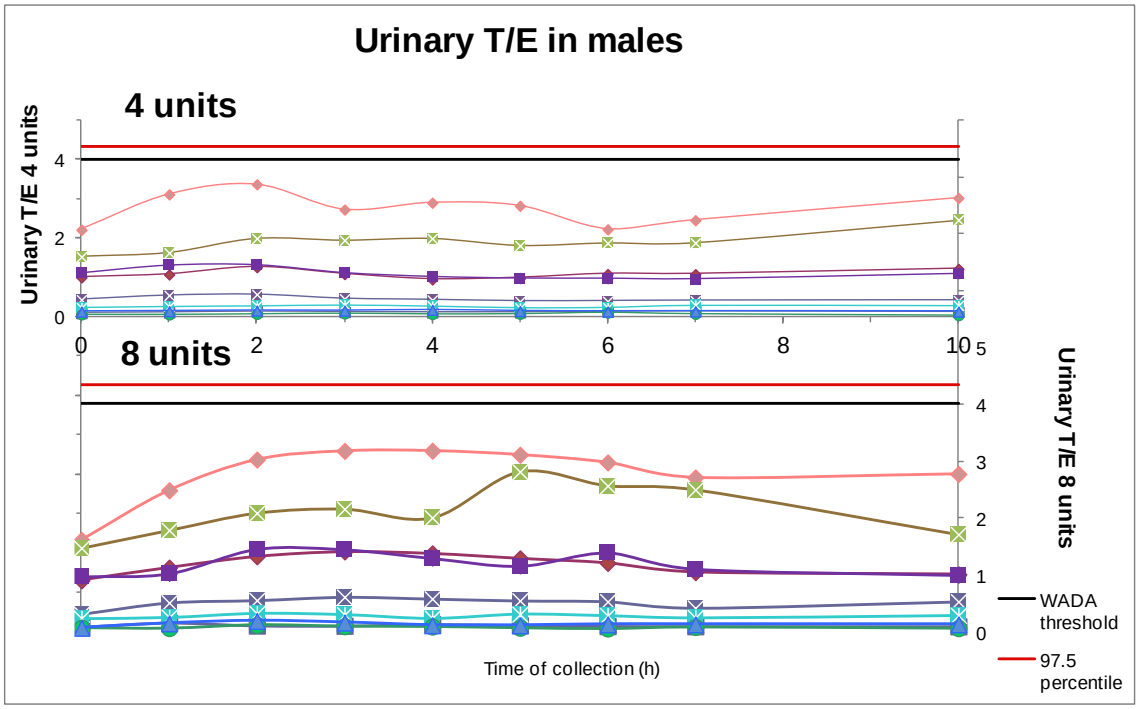


Figure 3.11 Urinary T/E in males, 4 and 8 units

The urinary T/E response is different for 4 and 8 units (p-value < 0.05) averaged over time and gender, and is also different for males and females (p <0.05) averaged over time and units, and these changes were significant over time (p <0.05) averaged over units and gender.

Regarding gender and units effects, averaged over time, females may have a higher T/E response but it is the same type of response when the units increase (p >0.05), i.e., 4 and 8 units have the same variation in T/E response for females and males. However, the time profile is different for females and males (p <0.05), averaged over gender, with the highest T/E for 4 units for women at 3 h and in men at 2 h, and for 8 units in females peak time was at 5 h whereas for men it was at 2 h.

There is also a statistically significant difference between time profiles for males and females, units combined (p <0.05).

In females, at 4 units, 4 females showed a T/E ≥2.65 without any reaching the WADA's action limit threshold of 4. At 8 units, 6 females had a peak T/E ≥2.65 and 4 showed a T/E ≥4, peaking at 5 h and decreasing afterwards until its values were closer to baseline at 10 h, completely reaching baseline at 24 h.

In males, for 4 and 8 units, their T/E ratio increased at 2 h without ever exceeding the WADA threshold or the 97.5th percentile.

A graph from the statistical software SPSS with the normalized data (proportion increase from pre-administration sample) may be found in Figure 3.12 and .

All the graphs from SPSS are shown herein with the estimated marginal means on the y-axis. This is the mean response for each factor, in our case the time interacting with the units of alcohol (4 or 8), adjusted for any other variable such as gender in the model used.

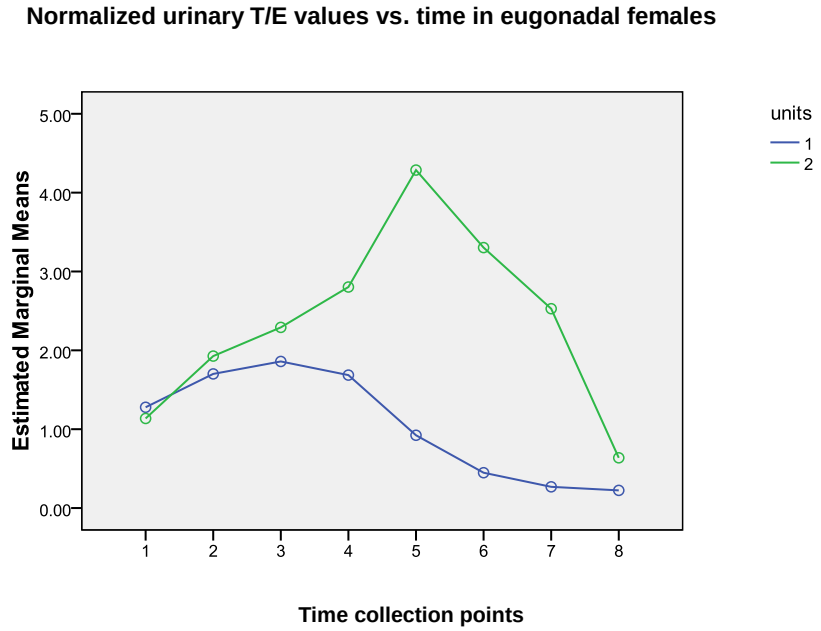


Figure 3.12 Normalized T/E in females. Units 1 = 4 units, 2 = 8 units

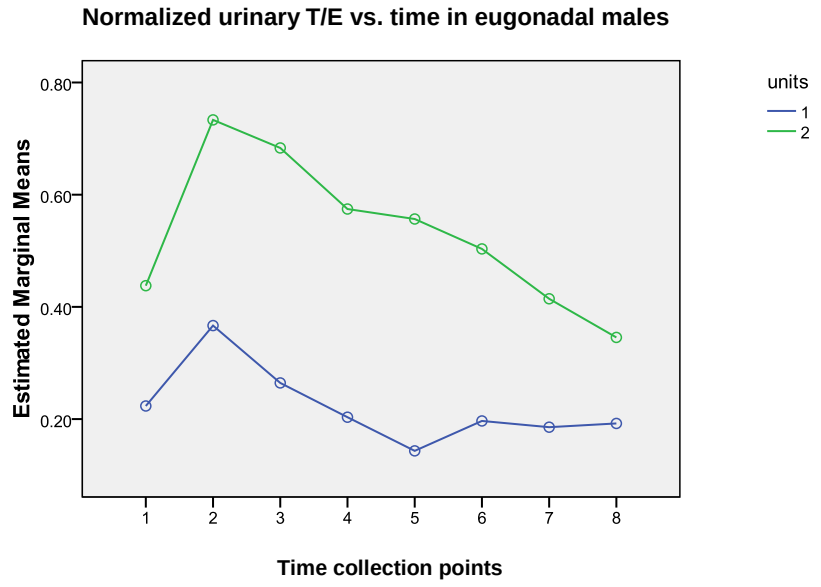


Figure 3.13 Normalized T/E in males. Units 1 = 4 units, 2 = 8 units

In males, the individual value for urinary T/E varies by less than 30 % and in females the variation up to 60 % may occur between the suspicious and unaffected sample (Kicman and Cowan, 2009, World Anti - Doping Agency, 2004).

To illustrate the amount of variation in the volunteers after 4 and 8 units of alcohol, the relative standard deviation was performed. To illustrate a 'real-life' sample with collections pre, during and post-administration, the samples used were pre-administration of alcohol (CTRL), average higher urinary T/E (for 4 and 8 units in males at 2 h, after 4 units in females at 3 h and after 8 units at 5 h), and the 48 h sample (Table 3.20).

If they vary by more than 30 % in males and 60 % in females, then that could be indicative of an individual that has discontinued T administration.

Table 3.20 Individual variation in females and males, after 4 units (32 g) and 8 units (64 g) of alcohol

	Females		Males	
	4 units	**8 units**	**4 units**	**8 units**
CTRL	0.7	0.7	0.8	0.6
High T/E	2.1	3.4	0.9	0.8
48 h	0.7	0.6	0.7	0.6
Mean	1.2	1.6	0.8	0.7
SD	0.8	1.5	0.1	0.1
RSD %	**68.4**	**98.8**	**13.3**	**17.7**

The individual variation in males after 4 and 8 units did not exceed 30 %. However, in females the variation in urinary T/E was 68 % after 4 units and 99 % after 8 units, which in both cases exceeds the 60 % variation expected in females.

3.3.6.2 Urinary T concentration

Samples that have a T and E concentration higher than 200 ng/mL are considered suspect by WADA standards and submitted to further analysis by GC-IR-MS (World Anti-Doping Agency, 2004).

The 97.5th percentile for T concentration for females was measured to be 29.4 ng/mL and for males it was 103 ng/mL (Van Renterghem et al., 2010).

Due to the difference in females between the reference value and the WADA threshold it is clearer to present separate graphs rather than combined ones as in the previous section. Therefore, urinary T concentration for females 4 units may be found in Figure 3.14 and for 8 units in Figure 3.15, followed by the male data.

All the concentrations presented have been adjusted according to their SG.

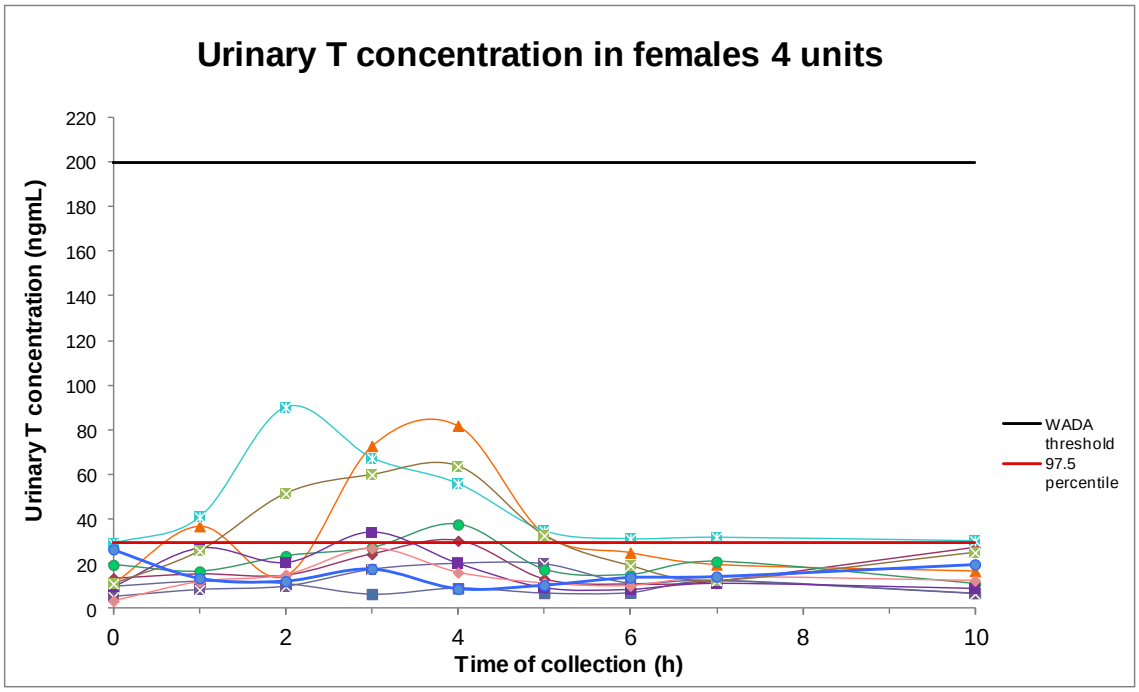


Figure 3.14 Urinary T concentration (ng/mL) in females, 4 units

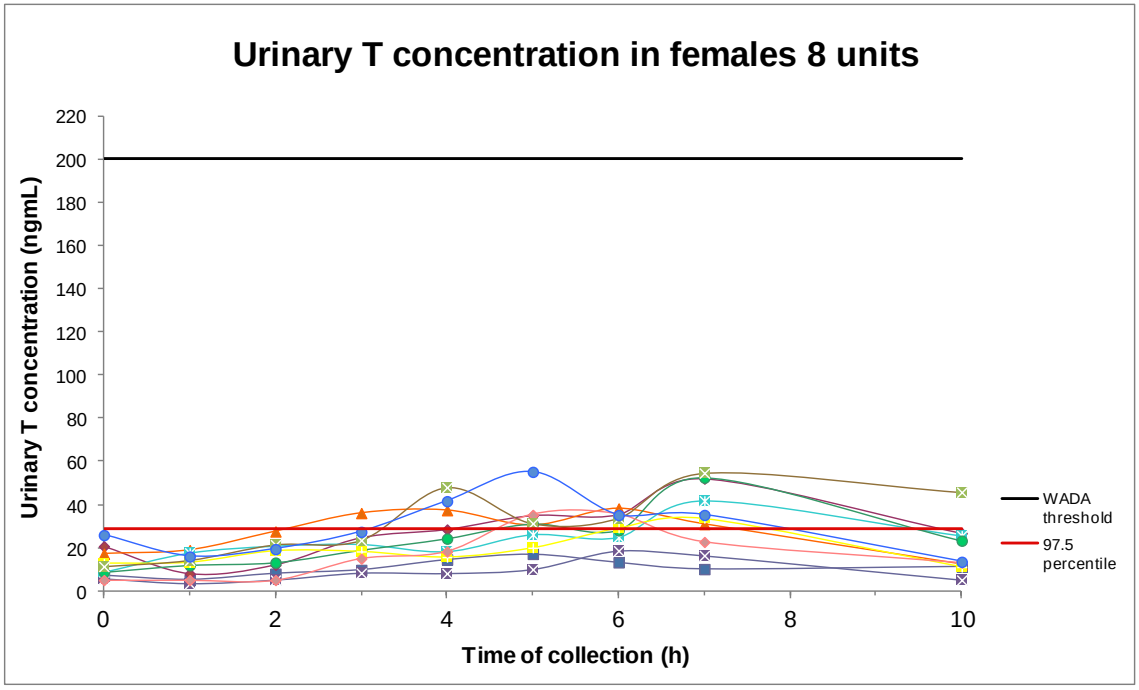


Figure 3.15 Urinary T concentration (ng/mL) in females, 8 units

In males, the urinary T concentration for 4 units may be found in Figure 3.16 and for 8 units in Figure 3.17.

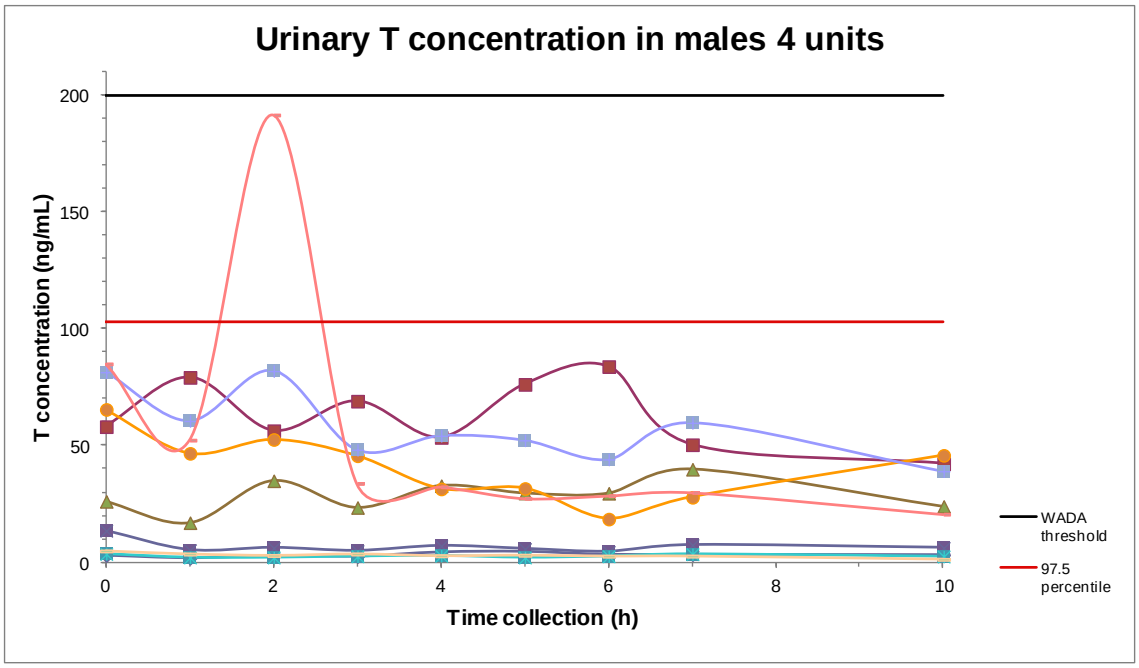


Figure 3.16 Urinary T concentration (ng/mL) in males, 4 units

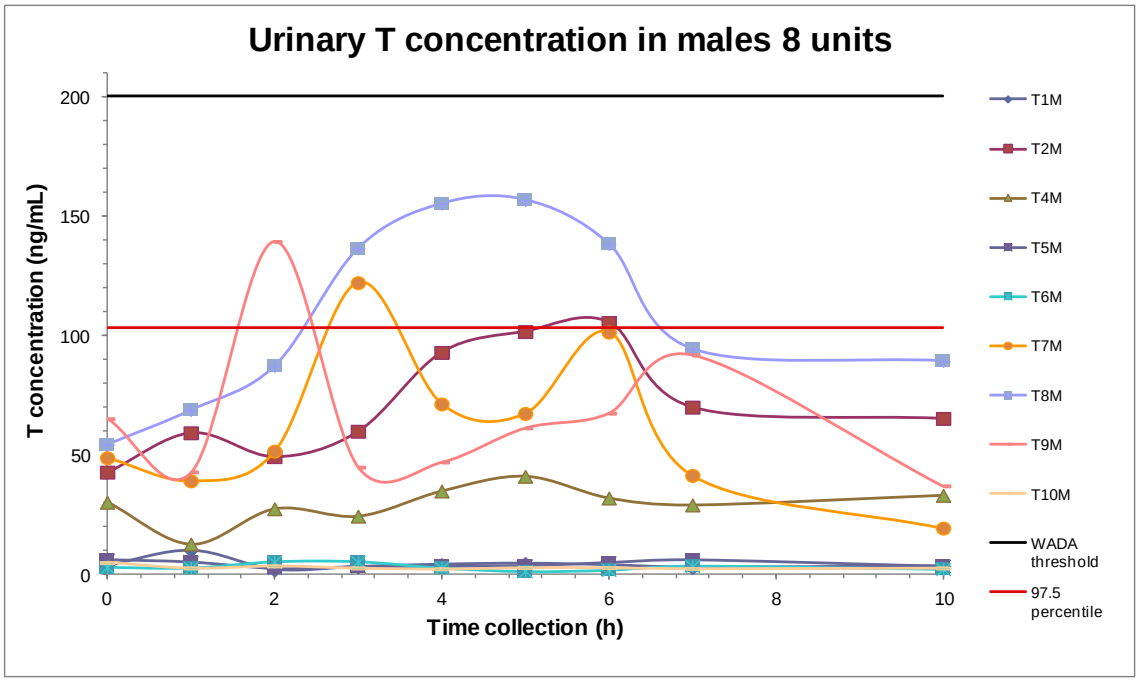


Figure 3.17 Urinary T concentration (ng/mL) in males, 8 units

The urinary T concentration response is the same for 4 and 8 units ($p > 0.05$), averaged over time and gender and it is also different from female to male ($p < 0.05$) averaged over time and units. The females may have a higher response but it does not appear to change when the units increase ($p > 0.05$), averaged over time.

There were changes over time in the T concentration ($p < 0.05$), averaged over units and gender. The concentration significantly increased over time after 4 to 8 units ($p < 0.05$) averaged over gender, with the highest T concentration for 4 units in females at 3 h and males at 2 h, and for 8 units in females at 7 h and in males at 6 h. A different time profile was observed for female and male (units combined) with a p-value <0.05.

None of the volunteers reached the WADA threshold, but 6 females and 1 male went above the 97.5th percentile in the 4 units intake; and 7 females and 4 males in the 8 units intake.

The normalized data may be found in Figure 3.18 and.Figure 3.19.

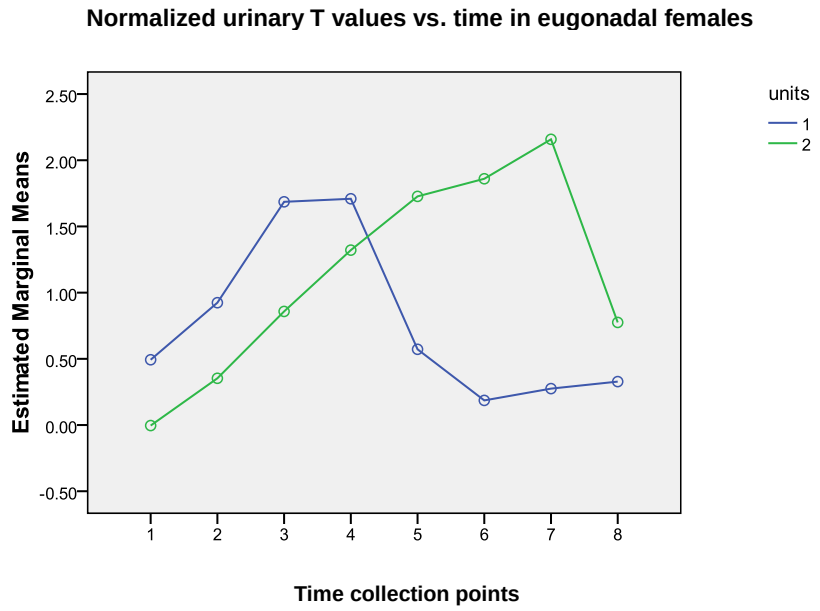


Figure 3.18 Normalized urinary T values in females. Units 1 = 4 units, 2 = 8 units

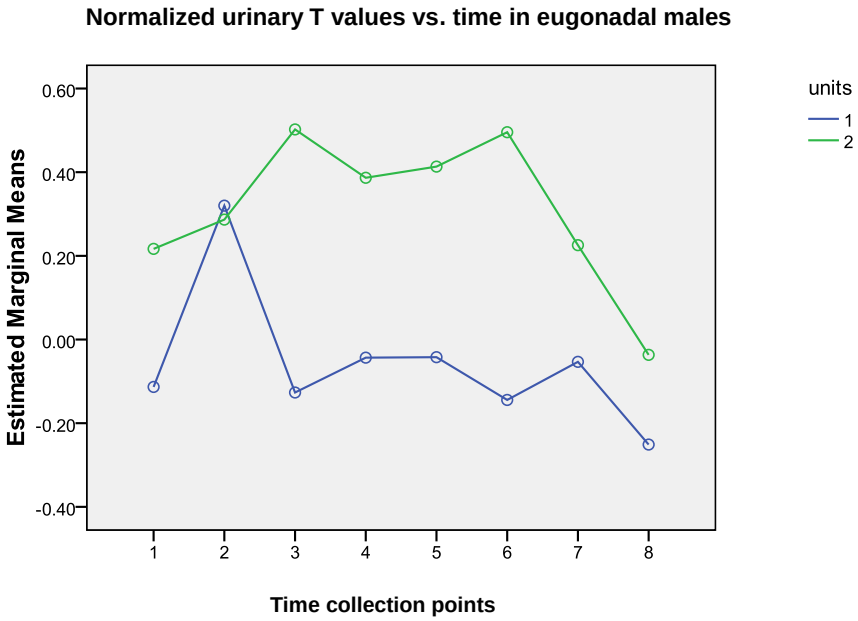


Figure 3.19 Normalized urinary T values in males. Units 1 = 4 units, 2 = 8 units

3.3.6.3 Urinary E concentration

The 97.5th percentile for E concentration in females is 28.5 ng/mL, and for males it is 88.9 ng/mL (Van Renterghem et al., 2010). The WADA threshold is 200 ng/mL for both females and males.

The plotted raw concentrations may be found in Figure 3.20 for females 4 units and in Figure 3.21 for 8 units.

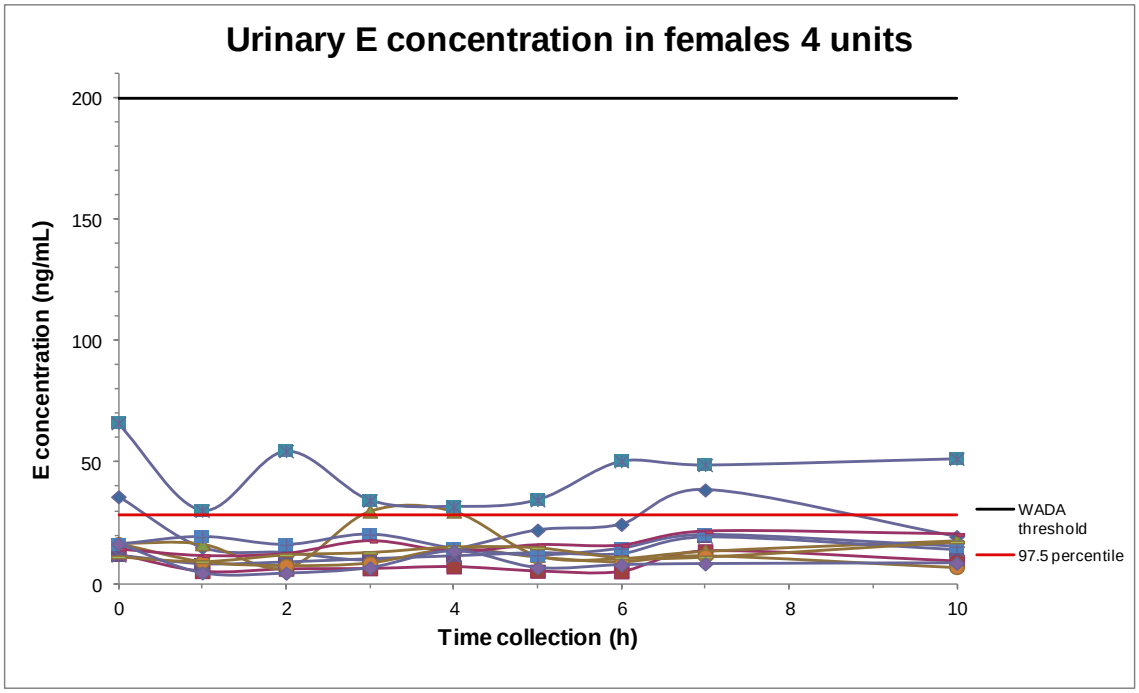


Figure 3.20 Urinary E concentration (ng/mL) in females, 4 units

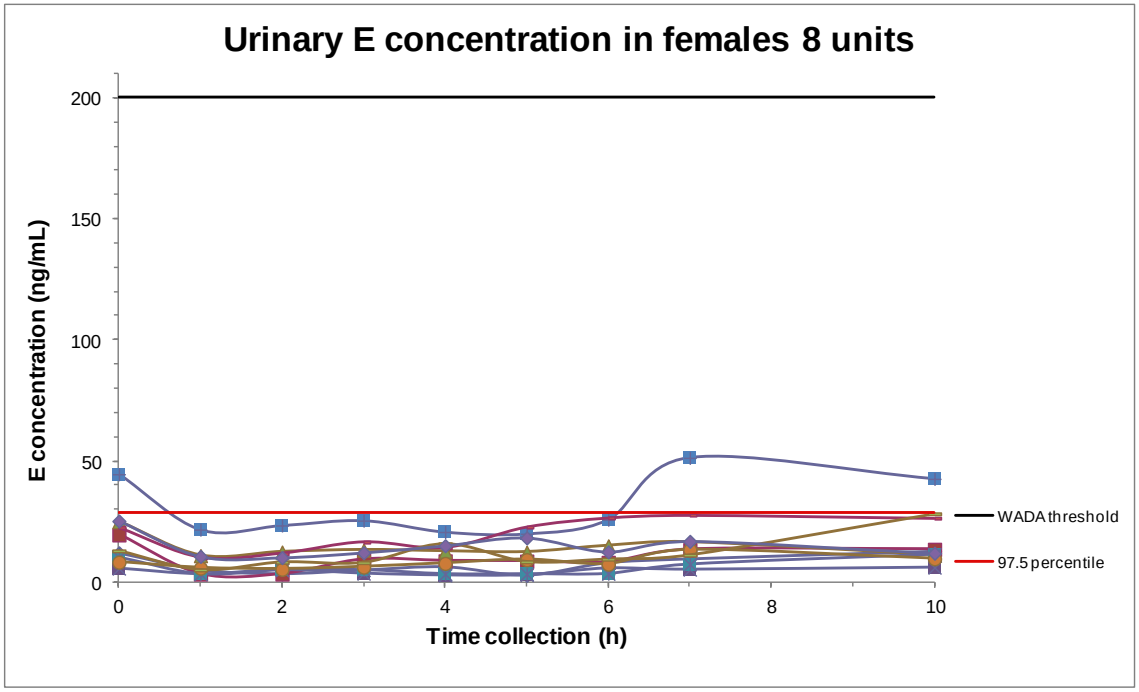


Figure 3.21 Urinary E concentration (ng/mL) in females, 8 units

For males, the data may be found in Figure 3.22 for 4 units and in Figure 3.23 for 8 units.

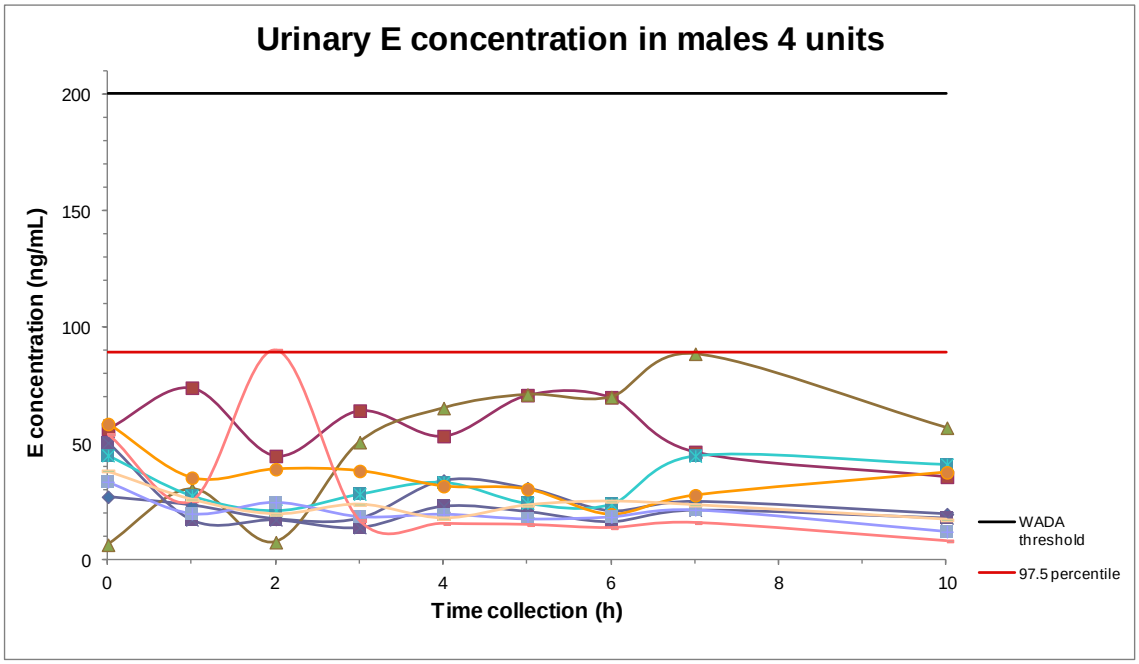


Figure 3.22 Urinary E concentration (ng/mL) for males, 4 units

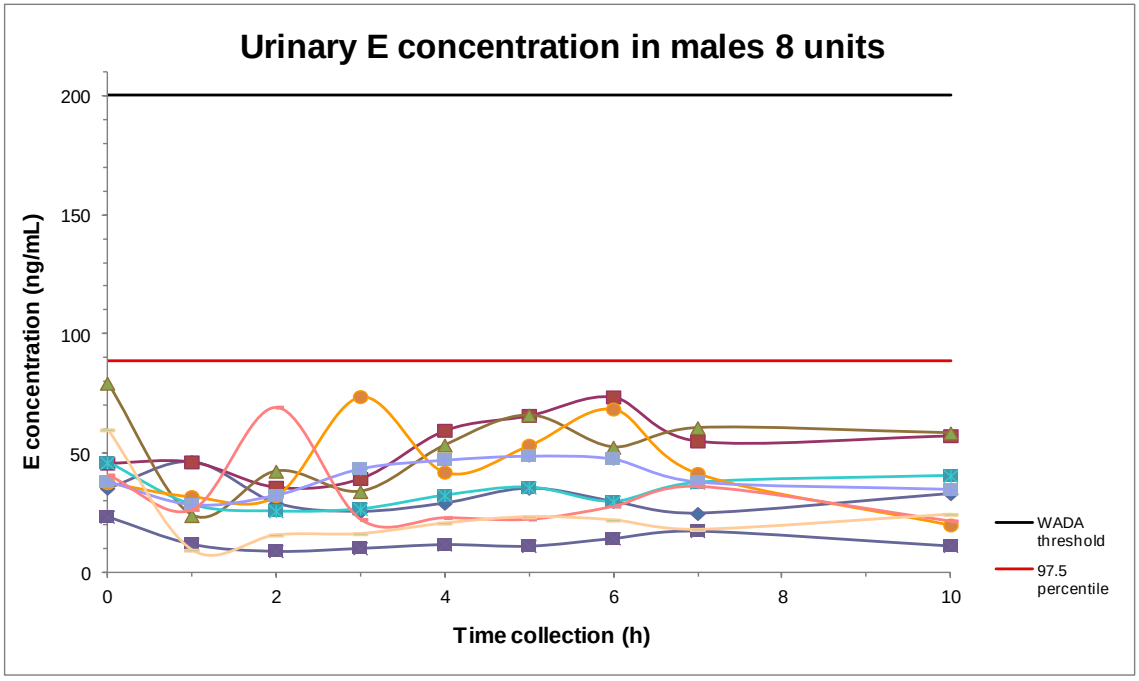


Figure 3.23 Urinary E concentration (ng/mL) for males, 8 units

With respect to urinary E concentration, no difference in average response for 4 or 8 units ($p > 0.05$), averaged over time and gender was observed, nor for female and male ($p > 0.05$). The males may have a higher response but it stays the same when the units increase averaged over time and units ($p > 0.05$).

There were changes over time in the E concentration ($p < 0.05$) averaged over units and gender. The E concentration decreased albeit not significantly over time for 4 and 8 units ($p > 0.05$) averaged over gender, with the lowest E concentration for 4 and 8 units in females being at 1 h, and with males at 1 h after 4 units and 3 h after 8 units.

None of the volunteers went above the WADA threshold, but 3 females and 1 male in 4 units and 2 females in 8 units went above the 97.5th percentile.

Normalized mean values for urinary E may be found in Figure 3.24 and Figure 3.25.

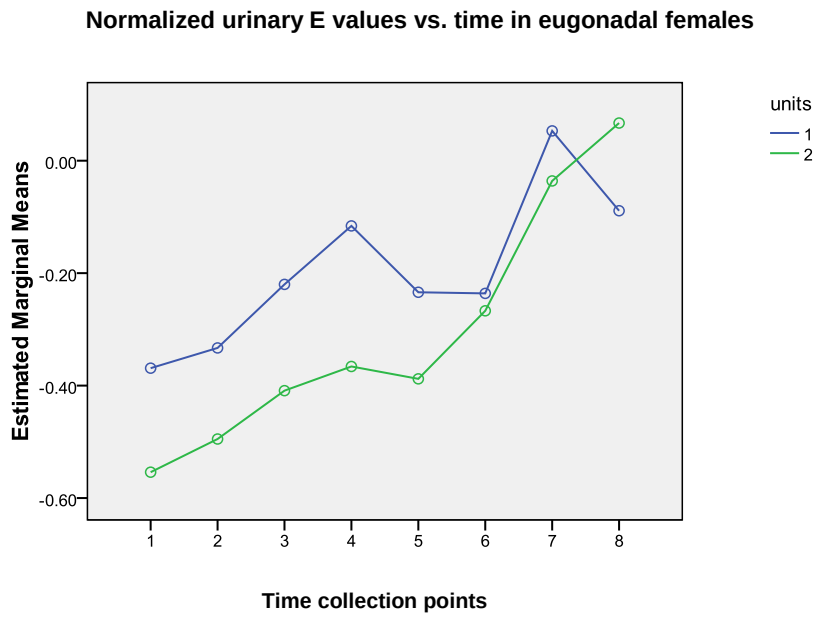


Figure 3.24 Normalized urinary E values in females. Units 1 = 4 units, 2 = 8 units

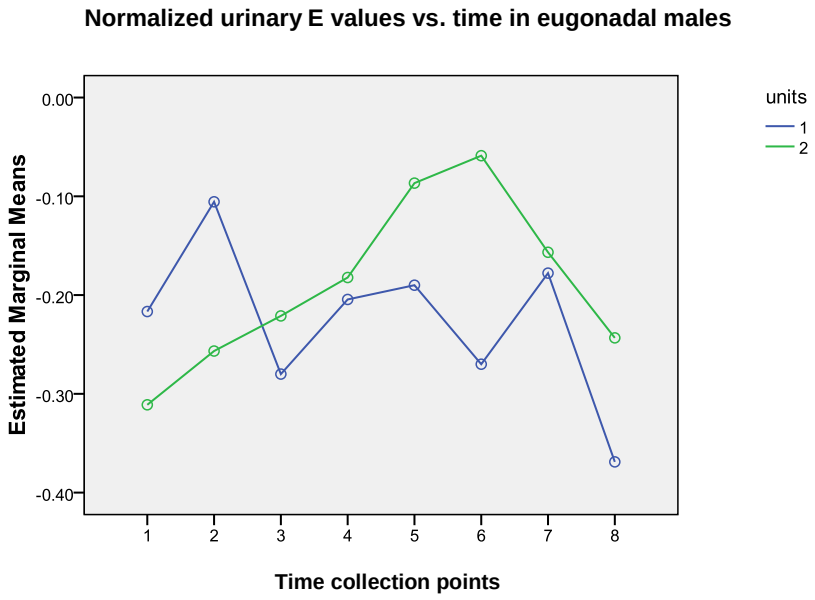


Figure 3.25 Normalized urinary E values in males. Units 1 = 4 units, 2 = 8 units

3.3.6.4 Urinary 5α-DHT concentration

The 97.5th percentile for 5α-DHT concentration in females was 20.5 ng/mL, and for males it was 21.5 ng/mL (Van Renterghem et al., 2010).

To date, WADA has not established a threshold for the urinary concentration of 5α-DHT.

The concentrations for females at 4 units may be found in Figure 3.26, and for 8 units in Figure 3.27.

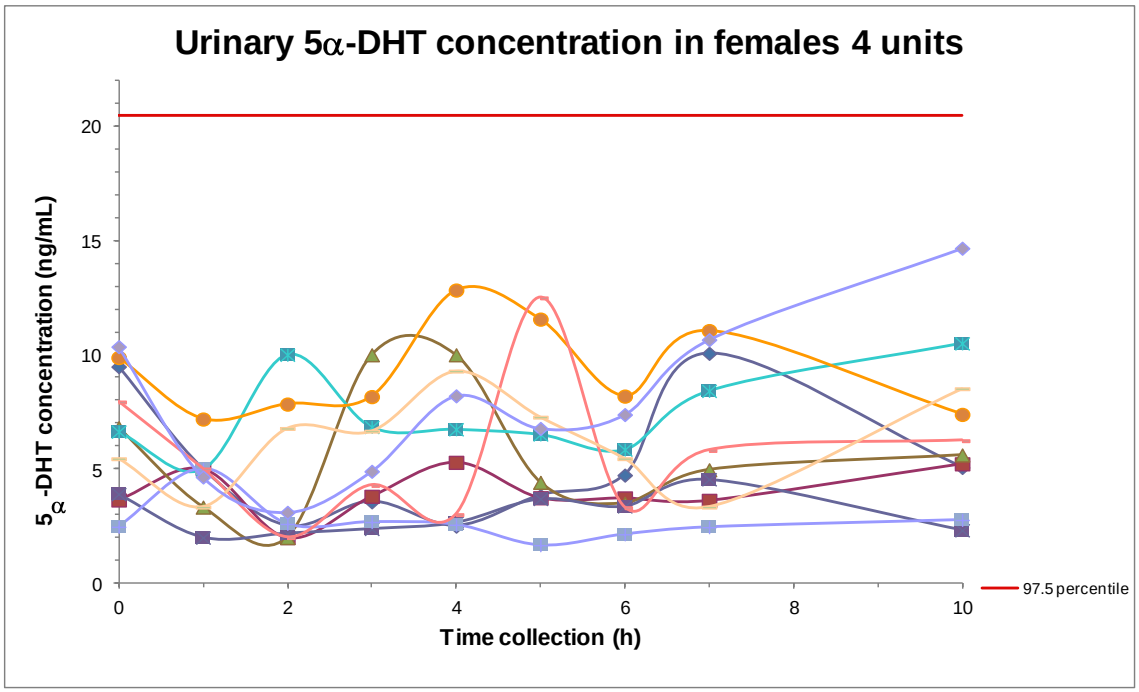


Figure 3.26 Urinary 5α-DHT concentration (ng/mL) for females, 4 units

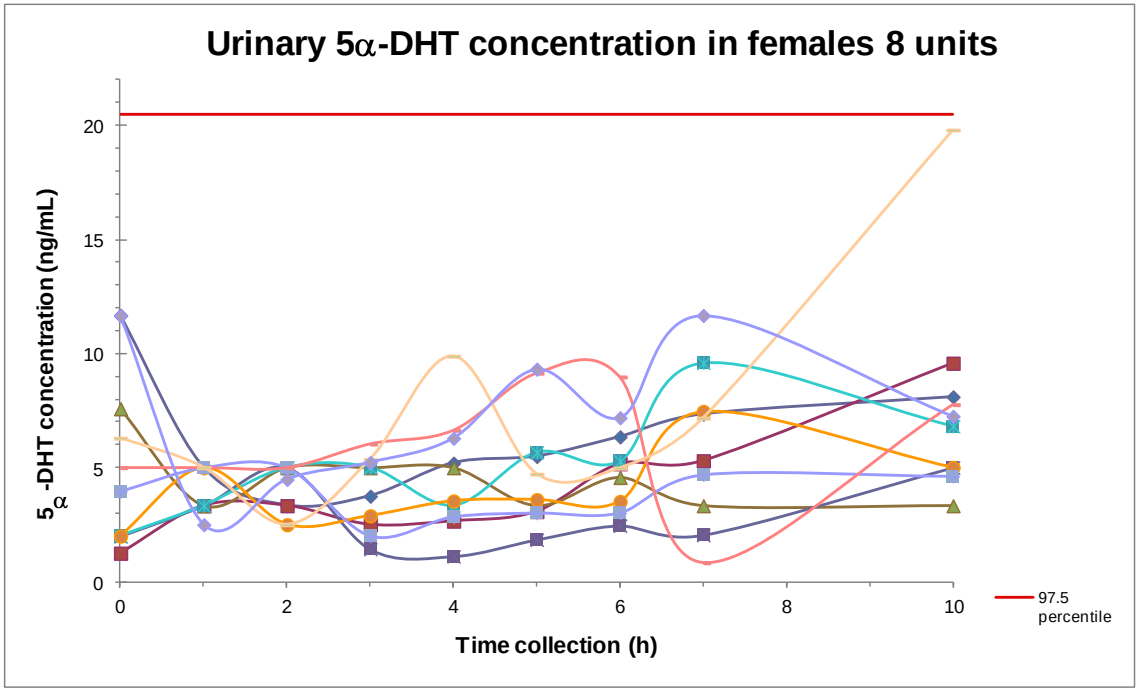


Figure 3.27 Urinary 5α-DHT concentration (ng/mL) for females, 8 units

In males, for the ingestion of 4 units of alcohol the results may be found in Figure 3.28, and for 8 units in Figure 3.29.

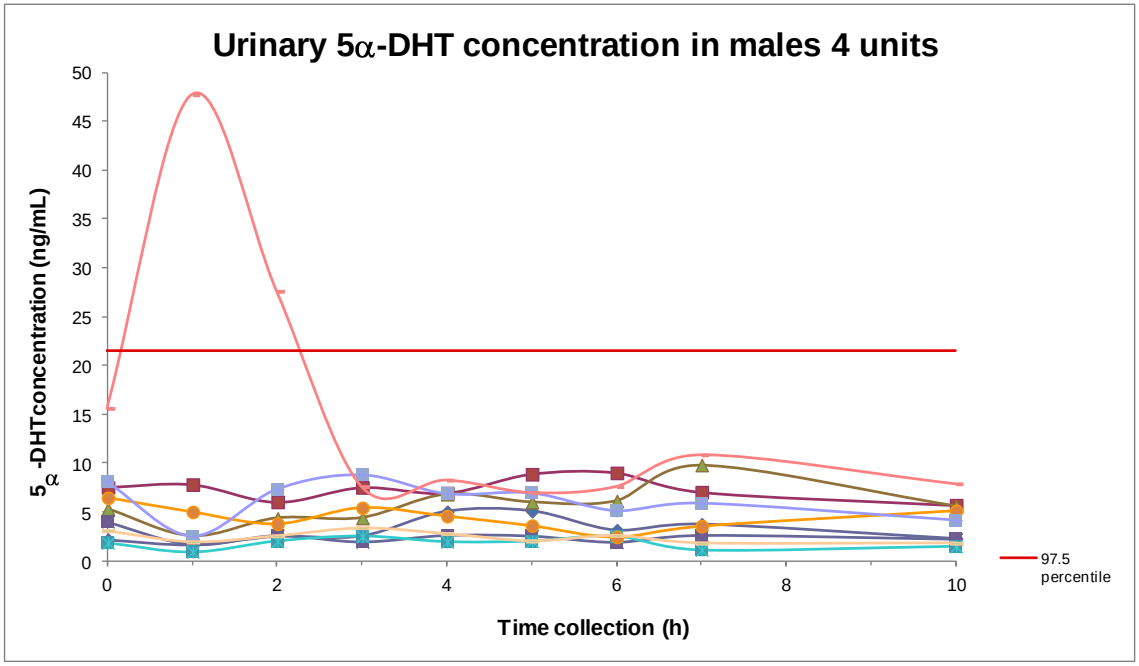


Figure 3.28 Urinary 5α-DHT concentration (ng/mL) in males, 4 units

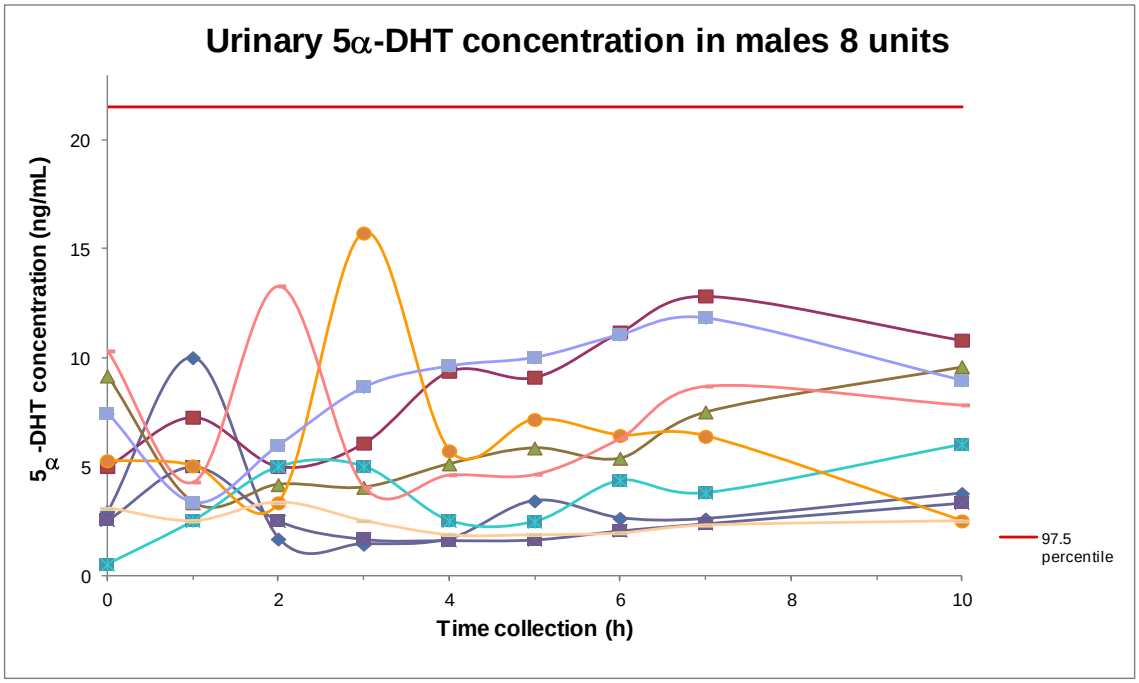


Figure 3.29 Urinary 5α-DHT concentration (ng/mL) in males, 8 units

With 5α-DHT concentration, the response is the same for 4 and 8 units ($p>0.05$) averaged over time and gender, and the same for females and males ($p >0.05$) averaged over time and units.

The males may have a higher response but it stays the same when the units increase ($p > 0.05$) averaged over time. There were no changes over time in the 5α-DHT concentration ($p > 0.05$) averaged over units and gender. The 5α-DHT concentration changes over time for 4 and 8 units ($p < 0.05$) averaged over gender, with the highest 5α-DHT concentration for 4 units in females at 4 h and 10 h and for males at 4 h; and 8 units for females and males at 10 h. The same time profile was observed for female and male (units combined) with a p-value >0.05.

Only one male volunteer, 4 units, went above the 97.5th percentile after 1 h.

The normalized the 5α-DHT values may be found in Figure 3.30 and Figure 3.31.

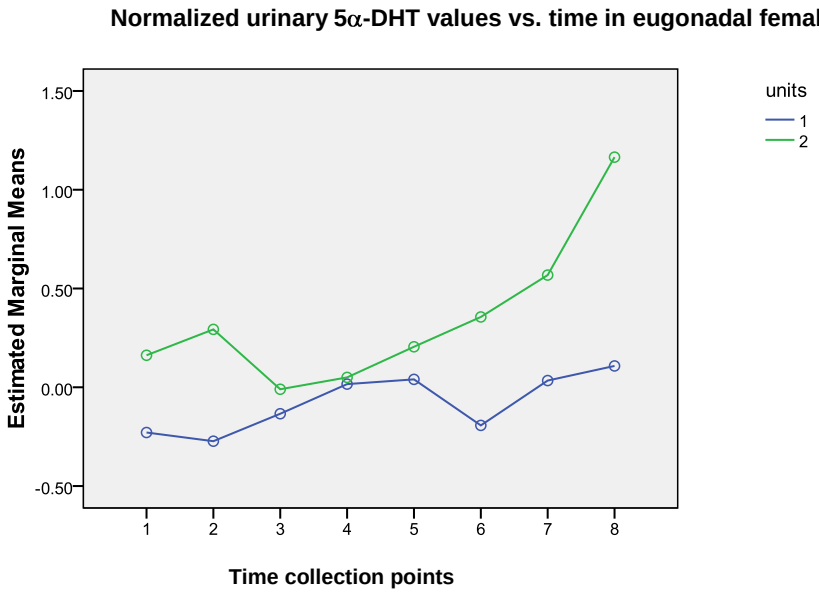


Figure 3.30 Normalized urinary 5α-DHT values in females. Units 1 = 4 units, 2 = 8 units

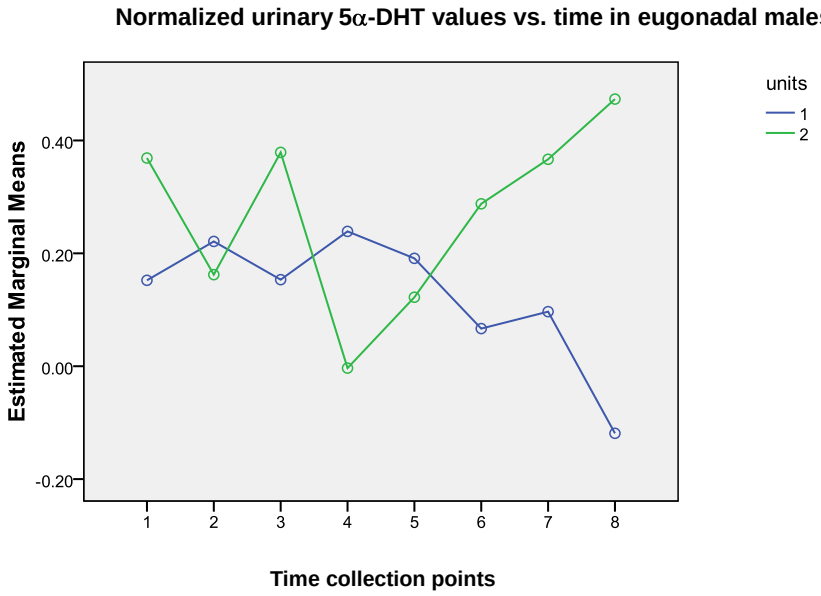


Figure 3.31 Normalized urinary 5α-DHT values in males. Units 1 = 4 units, 2 = 8 units

In summary, both 4 and 8 units of alcohol administration cause an increase in T/E in both males and females with the increase being more accentuated in females and the time profiles varying depending on gender.

With respect to SG adjusted T concentration, there were significant changes over time with females being more affected than men (higher increase in urinary T concentration)

The urinary E concentration decreased over time due to alcohol. However, unlike T concentration, females and males are not affected differently.

With urinary 5α-DHT, a product of T biosynthesis, there is a significant increase over time after 4 and 8 units of alcohol, averaged over gender. Females and males are affected similarly, and the time profiles for each gender were not significantly different.

3.3.6.5 Urinary EtG concentrations

Concentrations of alcohol's biomarkers (EtG and EtS) determined using LC-MS/MS were kindly provided by Dr. Alfonso Lostia's project (submitted to Alcohol and Alcoholism). In brief, deuterated d_5-EtG was added to the sample, QCs and standards and they were centrifuged at

10,000 g for 5 min to remove sediments. A simple 1:10 dilution in mobile phase (99 % formic acid 0.1% / 1% acetonitrile) was performed and the sample was injected at a flow rate of 200 µL/min using a validated ultra performance liquid chromatography-mass spectrometry (UPLC®-MS/MS) method. The transitions monitored were for the quantification of EtG was the precursor/product ion pair *m/z* 221 / 85 and the pair at *m/z* 125 / 80 for EtS.

The concentrations of these analytes were investigated to determine possible correlation with the increase in urinary T/E. These data are presented here and correlated with urinary T/E further down. All data was normalized against urine volume and presented as mg excreted/h since the urinary concentrations of EtG and EtS are affected by water intake. Statistical treatment was performed up to the collection of 10 h in 4 units, and 24 h in 8 units since the analytes were detectable until this time.

For females, a urinary EtG excretion rate time plot may be found in Figure 3.32 for 4 units, and for 8 units in Figure 3.33.

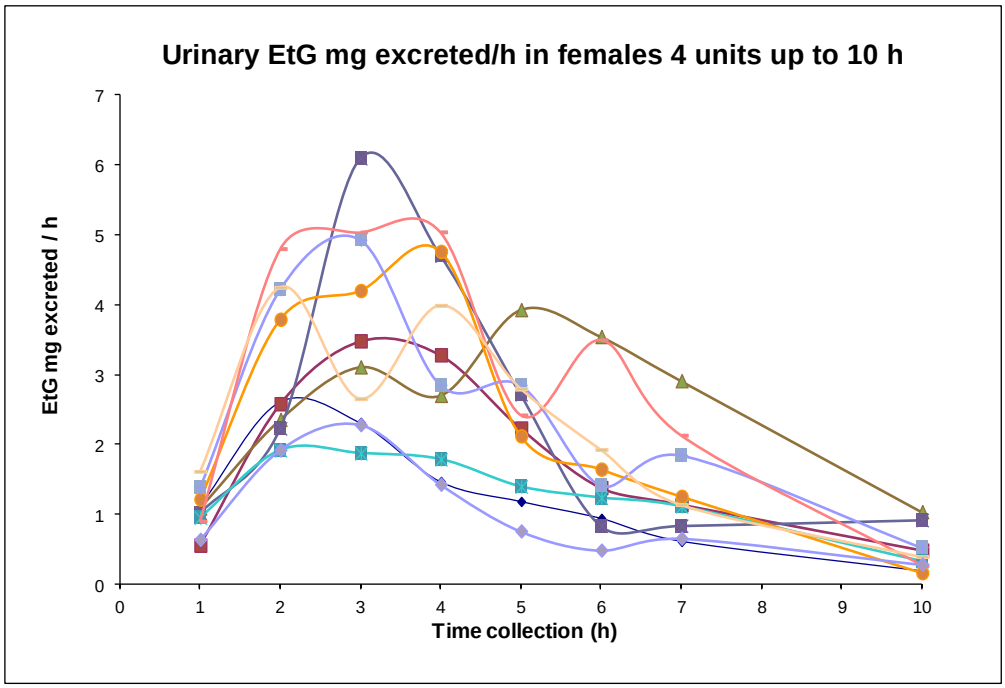


Figure 3.32 Urinary EtG mg excreted/h in females 4 units

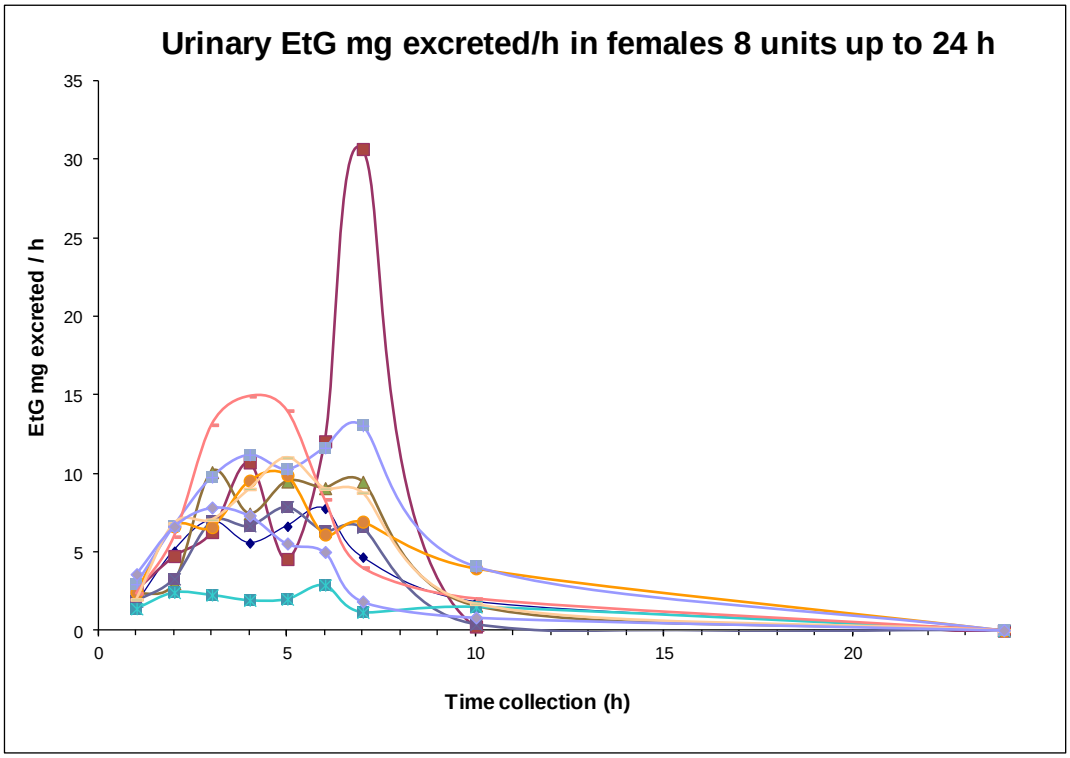


Figure 3.33 Urinary EtG mg excreted/h in females 8 units

In males, a urinary EtG excretion rate time plot may be found in Figure 3.34 for 4 units, and for 8 units in Figure 3.35.

It would appear that the elimination may be biphasic as is apparent with 3 of the volunteers (Figure 3.34).

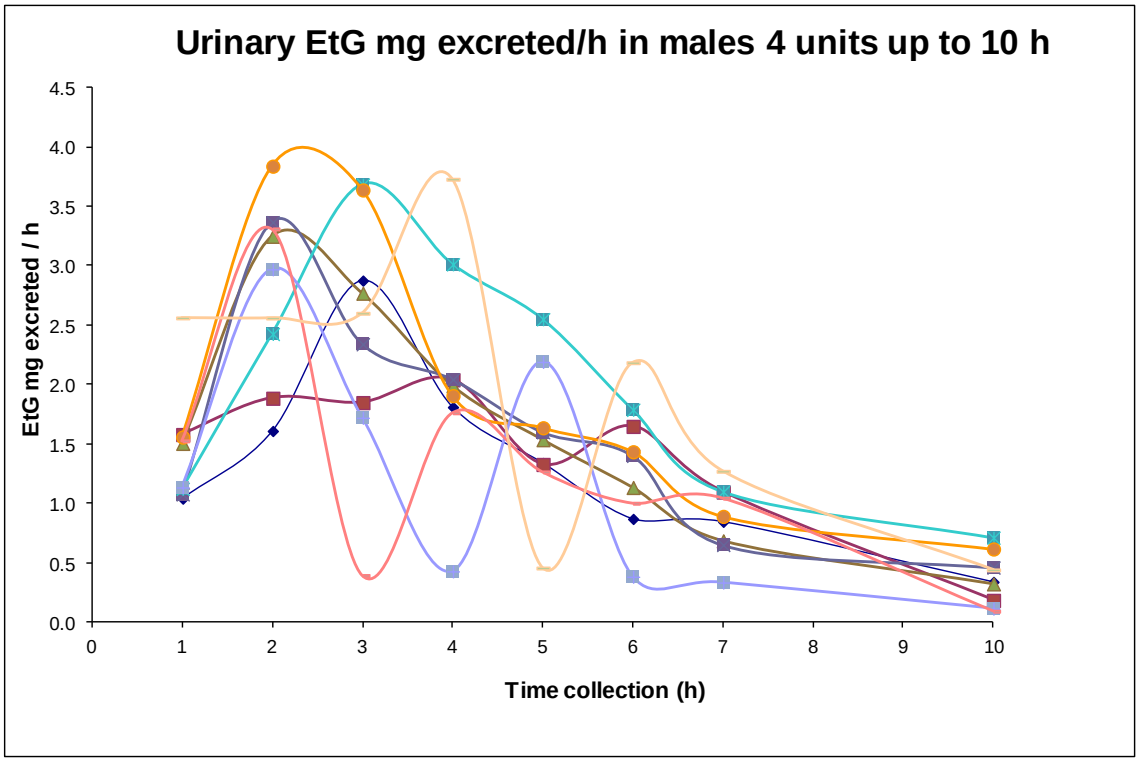


Figure 3.34 Urinary EtG mg excreted/h concentration in males 4 units

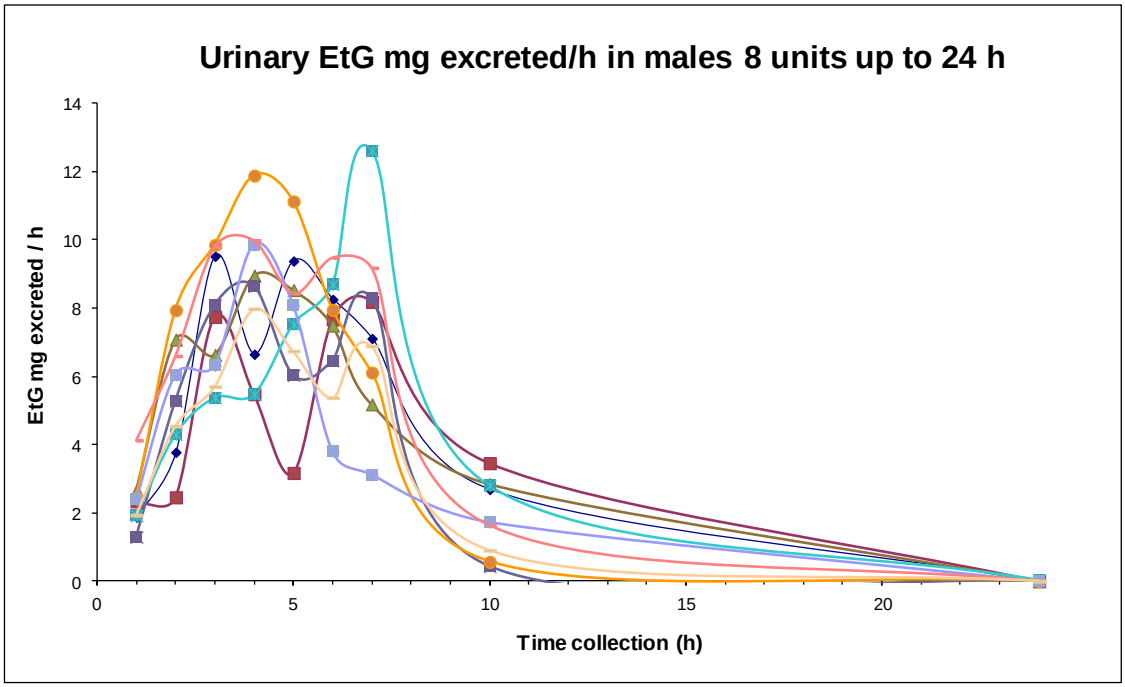


Figure 3.35 Urinary EtG mg excreted/h in males 8 units

Concerning the EtG urinary concentration, the response is different for 4 and 8 units, averaged over time and gender, with p <0.05; and different for females and males, averaged over time and units (p <0.05). EtG's response is higher in females but it is still the same type of response when the units increase, i.e., 4 and 8 units are the same for females and males (p=0.596), averaged over time.

There were changes over time in the EtG concentration (p <0.05) averaged over units and gender, and the time profile was different for 4 and 8 units (p <0.05), averaged over gender. The peak for 4 units was at 3 h and for 8 units, it peaked at 5 h. The same time profile was observed for female and male (units combined) with a p-value >0.05.

The difference between the time profiles for 4 and 8 units is the same for females and males (p >0.05).

3.3.6.6 Urinary EtS concentrations

For females a urinary EtS excretion rate time plot may be found in Figure 3.36 for 4 units, and for 8 units in Figure 3.37.

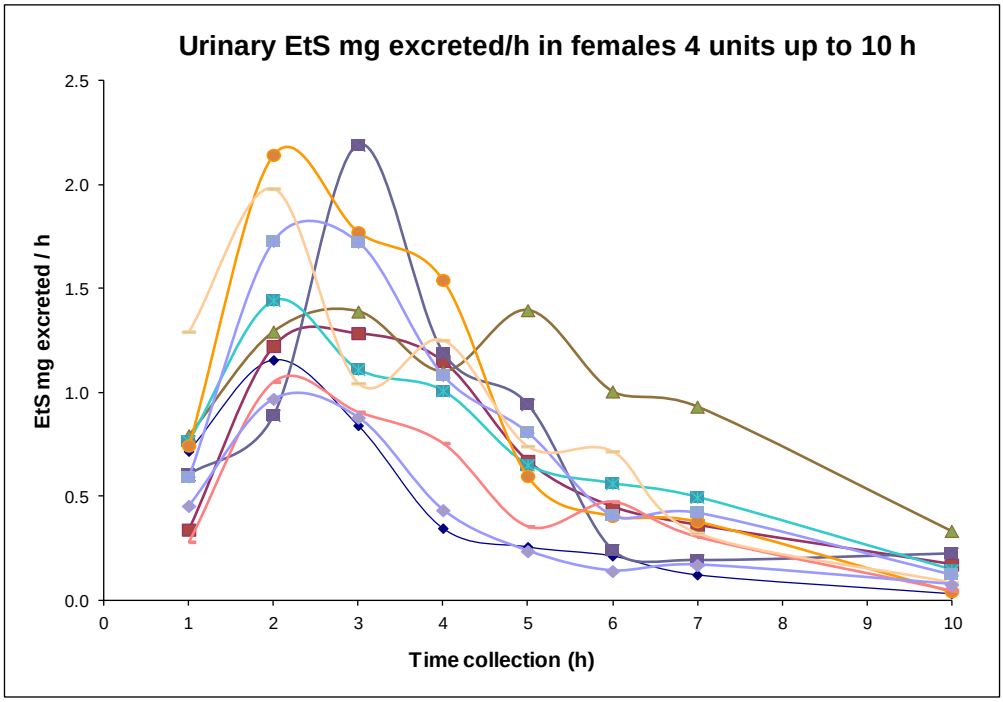


Figure 3.36 Urinary EtS mg excreted/h in females 4 units

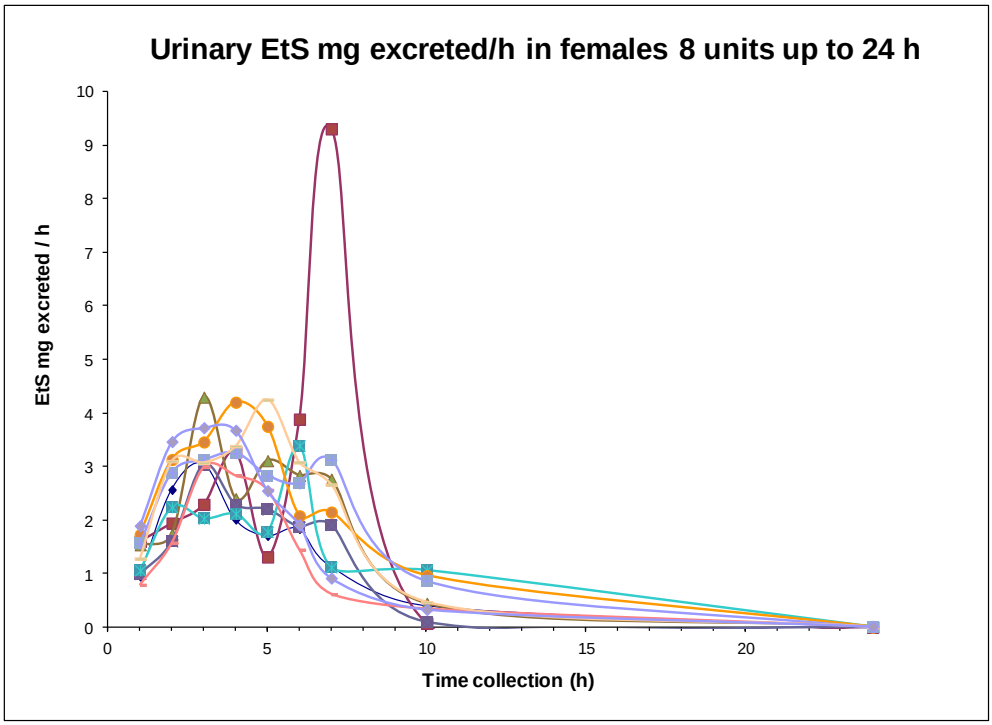


Figure 3.37 Urinary EtS mg excreted/h in females 8 units

For males, a urinary EtS excretion rate time plote may be found in Figure 3.38 for 4 units, and for 8 units in Figure 3.39.

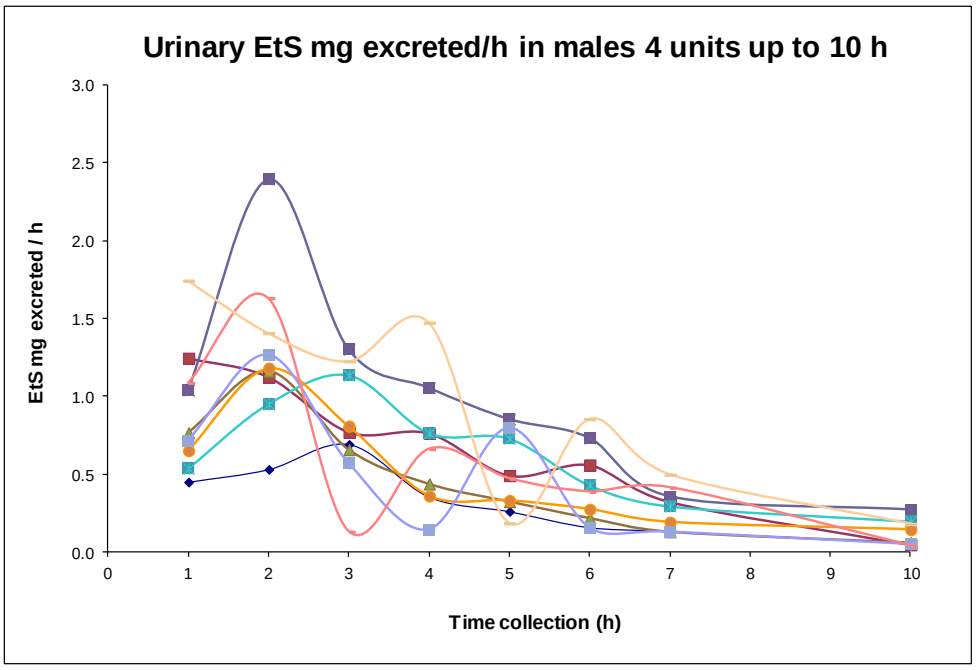


Figure 3.38 Urinary EtS mg excreted/h in males 4 units

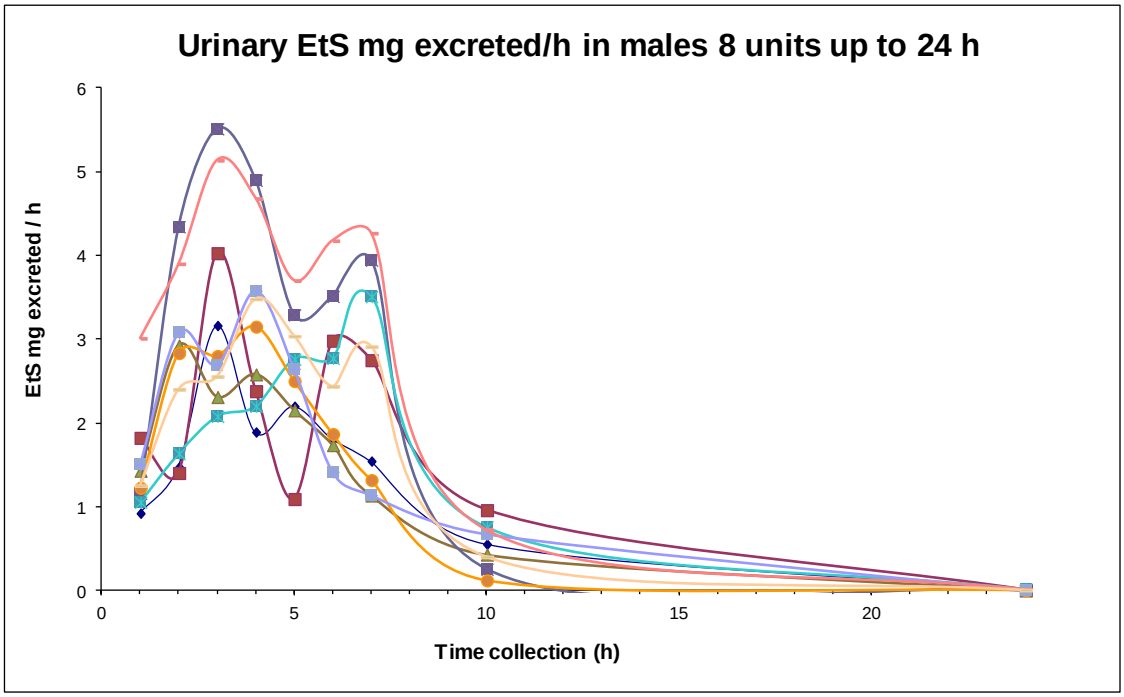


Figure 3.39 Urinary EtS mg excreted/h in males, 8 units

Regarding the EtS urinary concentration, the response is different for 4 and 8 units, averaged over time and gender, with $p < 0.05$; but with no difference being observed for females and males, averaged over time and units ($p > 0.05$). EtS's response is higher in females but it is still the same type of response when the units increase, i.e., 4 and 8 units are the same for females and males and males ($p > 0.05$), averaged over time.

There were changes over time in the EtS concentration ($p < 0.05$) averaged over units and gender, and the time profile was different for 4 and 8 units ($p < 0.05$), averaged over gender. The peak for 4 units was at 2 h and for 8 units, it peaked at 5 h. The same time profile was observed for female and male (units combined) with a p-value >0.05.

The difference between the time profiles for 4 and 8 units is the same for females and males ($p > 0.05$).

3.3.6.7 Urinary EtG/EtS

The urinary ratio of EtG/EtS was plotted for females 4 units and 8 units, and males 4 and 8 units and may be found in Figure 3.40.

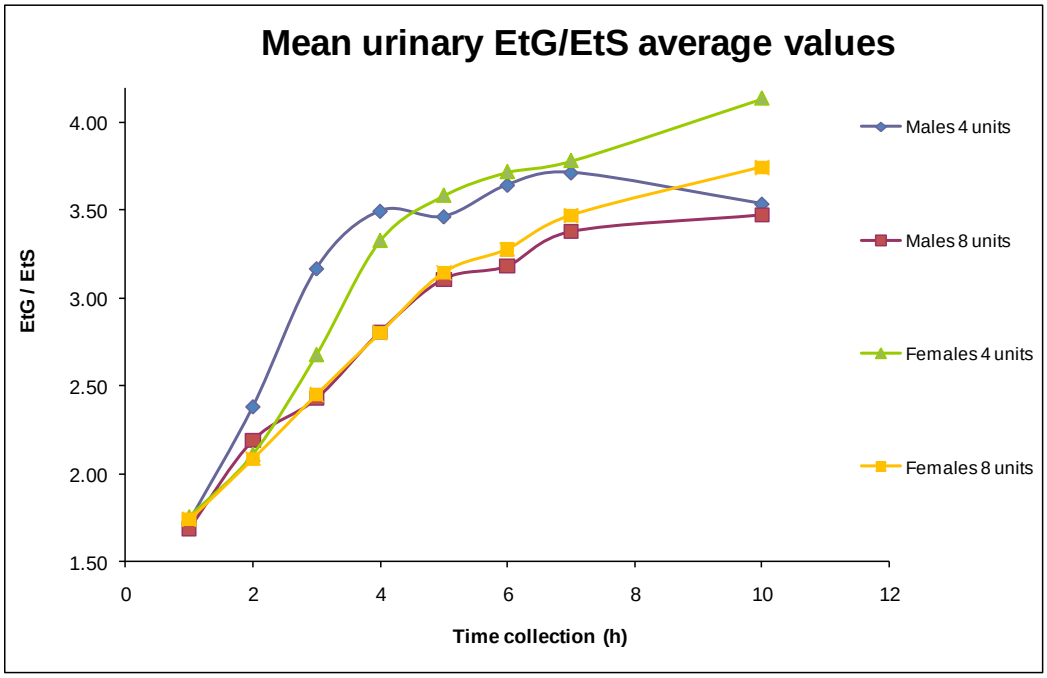


Figure 3.40 Mean urinary EtG/EtS for females (n=10) and males (n=9) at 4 and 8 units

With respect to EtG/EtS, a difference in response between 4 and 8 units, averaged over time and gender, was observed with $p < 0.05$; and it is the same for females and males, averaged over time and units ($p > 0.05$). EtG/EtS response is higher in females but it is still the same type of response when the units increase, i.e. 4 and 8 units are the same for females and males and males ($p > 0.05$), averaged over time.

There were changes over time in the EtG/EtS concentration ($p < 0.05$) averaged over units and gender, and the time profile was different for 4 and 8 units ($p < 0.05$), averaged over gender. The ratio increased in both units up to 7-10 h. The difference between the time profiles for 4 and 8 units is the same for females and males ($p > 0.05$).

EtG/EtS ratio increase in urine samples of all volunteers as a function of time. The ratio EtG/EtS is lower after 8 units than after 4 units in all volunteers.

In summary, the concentrations of EtG and EtS in urine behave almost similarly, except that EtG is gender dependant. With the 8 units intake, the urinary concentrations of both biomarkers increased. With EtG that increase was more than the double and with EtS was approximately double.

EtG/EtS increased as a function of time after alcohol administration.

3.3.7 Analysis of urinary T/E, EtG and EtS

The urinary T/E was averaged and plotted together with the urinary average EtG concentration and EtS concentration for females and males after 4 and 8 units.

The female data may be found below in Figure 3.41 and Figure 3.42.

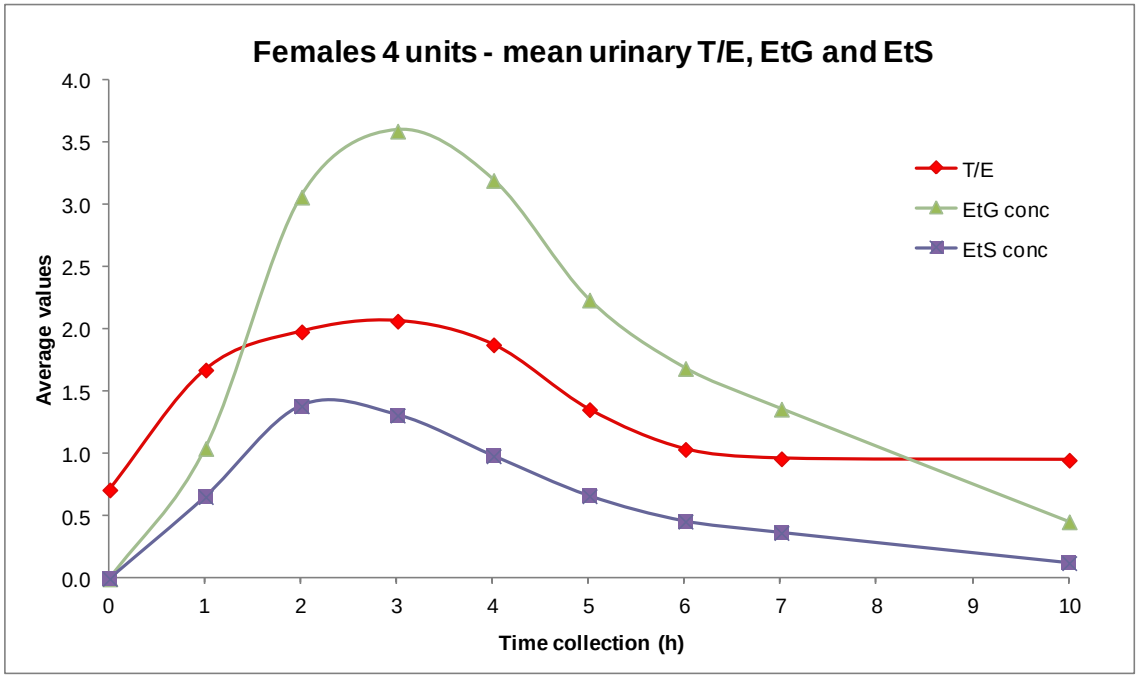


Figure 3.41 Mean urinary T/E plotted with urinary EtG and EtS (mg excreted/h), females 4 units (n=10)

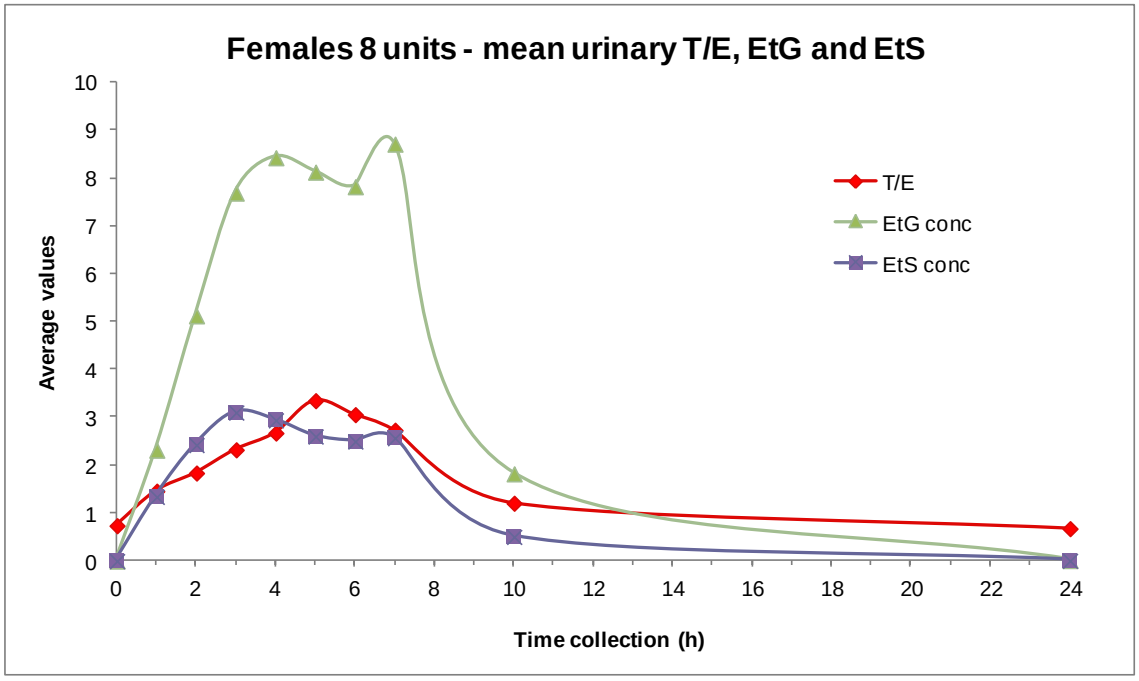


Figure 3.42 Mean urinary T/E plotted with urinary EtG and EtS (mg excreted/h), females 8 units

For males, and following the same analogy, mean T/E plotted with EtG and EtS concentrations for 4 units may be found in Figure 3.43. For 8 units please see Figure 3.44.

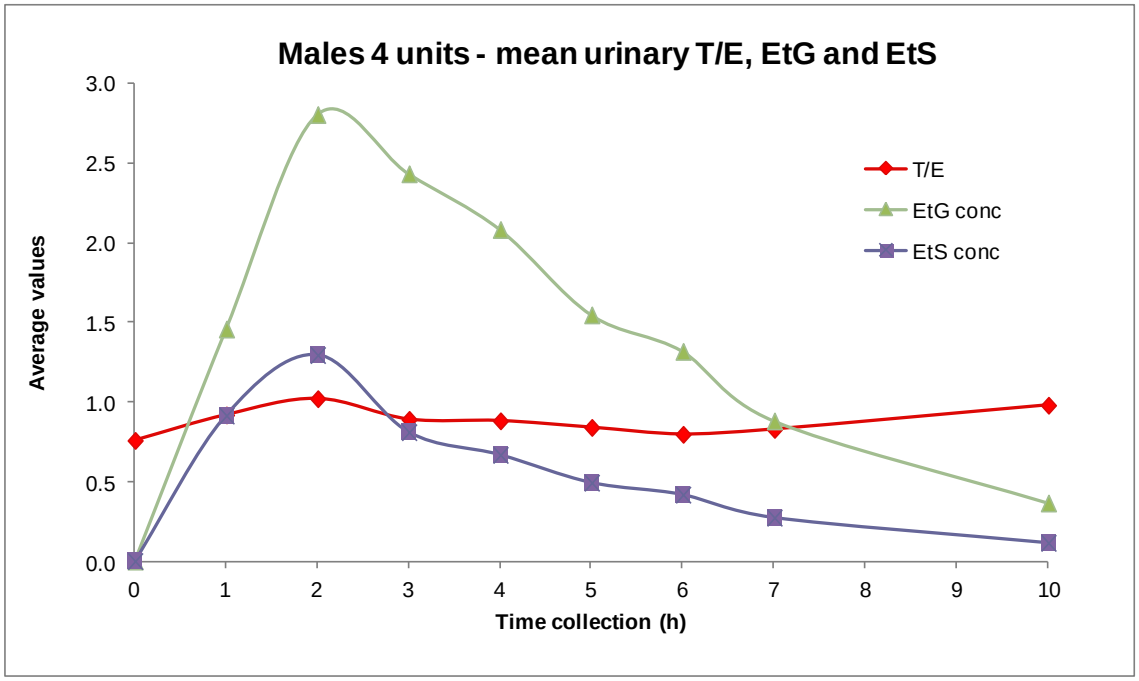


Figure 3.43 Mean urinary T/E plotted with urinary EtG and EtS (mg excreted/h), males 4 units

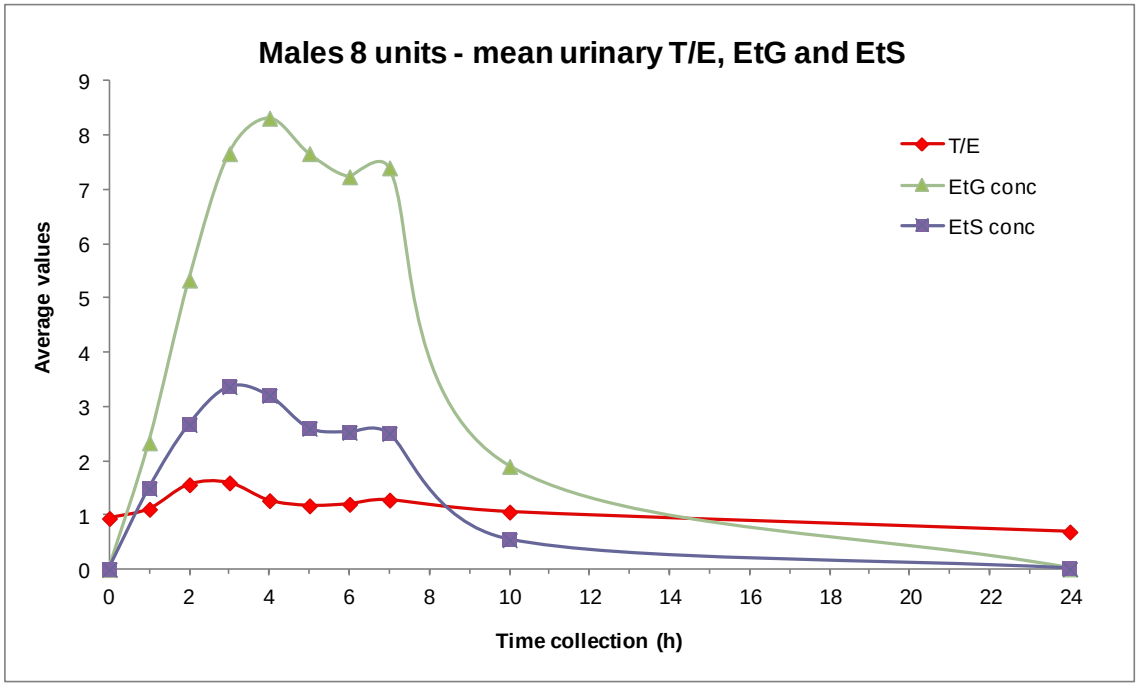


Figure 3.44 Mean urinary T/E plotted with EtG and EtS (mg excreted/h), males 8 units

The analysis of the previous graphs clearly shows that urinary T/E increases with the increase in the EtG and EtS mg excreted/h, as well as the EtG/EtS at least for the first two hours post-alcohol administration.

3.3.8 Correlation between urinary T/E and EtG mg excreted/h

A correlation between urinary T/E and EtG (one of alcohol's biomarkers) was performed.

In males, after 4 units, there was barely any correlation with no significant overall effect. After 8 units, there was an overall correlation because two of the volunteers had a very strong correlation whereas the remainder did not. In females, after 4 and 8 units there is a strong overall correlation which is statistically significant, but there are different individual responses. Some females responded more slowly to the increase in EtG concentration than did others. As an example, after 4 units, female volunteer T5F had a T/E of approximately 2 with an EtG excretion rate (mass per unit of time) of 2 mg/h whereas female volunteer T8F showed a T/E of approximately 1 with an EtG response of 5 mg/h. The same two volunteers, after 8 units, had a

similar behaviour. Volunteer T5F had a urinary T/E of 7 with an EtG of 2 mg/h volunteer T8F had an urinary T/E of 1.5 with a corresponding EtG concentration of 14 mg/h.

Urinary T/E correlated with EtG concentration in some individuals (n=x, $p<0.05$)..

If EtG were to be used as a marker of alcohol administration in doping in sports, it would be necessary to establish an individual threshold for each athlete. In order to do so, their response to alcohol at different units should be tested with hourly collection time and for a longer period of time.

3.4 Conclusions

This study showed that 4 and 8 units of alcohol increases urinary T/E significantly. This increase is more evidentially significant in females than in males, and the mean T/E profile (the mean of each volunteer's T/E per hour) is different between the genders as well as with the different units, with peak times changing from 3 h at 4 units for 5 h at 8 units.

The reason why it affects females more than males is perhaps due to the fact that females produce less T and, probably more importantly, less E appears in urine than men, and any perturbances in that mechanism will disturb the T/E.

In our study, the 4 units of alcohol were approximately 0.5 g/kg of alcohol and the 8 units were 1 g/kg. Although previous studies have been done where volunteers are administed alcohol, our study has a higher number of samples, being more representative of the population. Previous studies have also administered their volunteers with alcohol, and obtained high T/E values only after the administration of 2 g/kg of alcohol. Although there was an increase in T/E due to alcohol, it was not as high as the T/E we obtained in our study with only half the dose.

In females the highest T/E was 7.1, and in males it was 3.2. With a dose of 0.5 g/kg, Falk *et al.* had the highest T/E in males at 1.52; Karila *et al.* obtained the highest T/E in females at 4.4 and in males there was no increase due to alcohol but its highest T/E was 1.6; Mareck-Engelke *et al.* showed the highest T/E in females at 6.7 and in males at 2.4 (Falk et al., 1988, Karila et al., 1996, Mareck-Engelke et al., 1996). Only Seppenwoolde-Waasdorp *et al.* administered the same dose as we did but obtained lower maximum T/E ratios, for females at 3.4 and for males at 1.2 (Seppenwoolde-Waasdorp et al., 1996).

This was possibly because we administered the alcohol to our volunteers during 1 h period and in previous studies this was done during a 3 h or 4 h period, therefore we managed to demonstrate a more acute intoxication, provoking a higher T/E ratio (Falk et al., 1988, Karila et al., 1996, Geyer et al., 1996, Seppenwoolde-Waasdorp et al., 1996, Mareck-Engelke et al., 1996). Another reason may be because we have have a more sensitive analysis technique.

One study has related the increase in T/E with the increase in urinary EtG concentration (Grosse et al., 2009), however this was performed only in 4 males aged between 32-50. Our

study also correlates the increase of T/E with EtG showing that there is a subject based correlation.

The use of these biomarkers is important for doping in sports to avoid that athlete's samples are flagged when their T/E increases in their biological passport (Sottas et al., 2010). The biological passport is a longitudinal study recorded for every athlete that includes a steroid profile comprising individual measurements of monitored steroid concentrations and ratios. When values go above the athlete's personal averaged values, the athlete is 'flagged' and asked to provide samples more frequently. Perhaps the introduction of the measurement of urinary EtG and/or EtS in the WADA procedures when a T/E sample is above the athlete's personal threshold, would prevent the athlete being flagged for the collection of more samples, thus saving time and money to the sports authorities.

Thus, this work has shown that the two doses of alcohol alter the urinary T/E in both males and females, with females (after 8 units) going above the WADA's action limit of 4. There was a significant increase over time in urinary T concentration, a decrease in urinary E concentration over time even if not significant, and a significant increase in urinary 5α-DHT concentration.

Urinary T/E increases with the increase in urinary normalized EtG and EtS (mg excreted/h).

In the following chapter we will verify if any increase in urinary T concentration could be caused by an increase in LH after alcohol administration.

Chapter 4 INFLUENCE OF ALCOHOL ADMINISTRATION ON LH IN SERUM AND IN URINE

4.1 Introduction

4.1.1 LH and doping in sports

In this chapter the hypothesis that ethanol administration increases LH concentration, thereby increasing urinary T concentration, is tested.

LH is part of a feedback mechanism comprising the hypothalamus, the anterior pituitary and the gonads (testes in men, ovaries in women).

In men, as described in Chapter 1, LH stimulates T production. Under normal conditions, a lowering of the circulating T concentration will result in a rise in GnRH and consequently LH and FSH and *vice versa* (Emanuele and Emanuele, 2001, Griffin and Wilson, 1992), thereby maintaining a steady state.

Administration of T suppresses LH production resulting in lower circulating LH and, although there will be a higher excretion of T, there will be a lower excretion of LH. A ratio of T/LH in urine may thus be a useful indicator of T administration. This is valid only for males, since LH secretion is suppressed in females with oral contraception. Analysis of urine from 120 healthy males has shown that the reference range for T/LH is between 1-150 nmol/IU and urinary LH concentration in healthy males between 0.5 – 20 IU/L (Drug Control Centre, 2010) as measured by DELFIA® hLH assay (PerkinElmer, Northwich, UK). More details on this type of assay may be found in section 4.1.4.

In a sports doping case (2005), the defendant with a T/E above the WADA threshold of 4, had a T concentration (adjusted by specific gravity, see Equation 4.1) of 257 ng/mL, above the WADA threshold of 200 ng/mL (World Anti - Doping Agency, 2004). Despite having a urinary T/LH within the reference range (45 nmol/IU), the athlete's urinary LH concentration was considered abnormal with a value of 28.9 IU/L.

The athlete's blood alcohol concentration was estimated to be 350 mg% based on uncorroborated statements of the total amount of alcohol that the athlete had drunk.

4.1.2 LH concentrations in urine and serum

Pre-menopausal women's cycle phase comprises of menstruation, follicular phase, ovulatory (or mid-cycle) and luteal phase. It is during ovulation that the concentration of LH and FSH rise and trigger ovulation, which occurs around the 14^{th} day in 28 day-cycles.

The urinary and serum LH concentrations for both men and women are displayed in the table below.

Table 4.1 LH urinary and serum concentration for males and females

Matrix	Male (IU/L)	Female (IU/L)	References
Urine	0.5-20	Follicular phase: 1-25 Mid-cycle: 15-95 Luteal phase: 2-20 Postmenopausal: 40-110	For males: (Drug Control Centre, 2010) For females: (Inter Science Institute, 1995)
Serum	1-8	Follicular phase: 1-12 Mid-cycle: 17-77 Luteal phase: 0-15 Postmenopausal: 11-40	For females and males: (National Institute of Health, 2009)

4.1.3 LH & Alcohol

A literature review found only two papers in which LH had been analysed after alcohol administration.

In one paper, the volunteers were females (n=9) and males (n=12) that had gone to the emergency room in the hospital suffering from acute alcohol intoxication. Their serum LH concentration when compared to a control group had not changed significantly (Frias et al., 2002).

In another study, with 4 females and 4 males to whom 1.2 g/kg and 2.0 g/kg of alcohol was administered in two separate occasions for a period of 5 h, showed that the serum LH concentration increased. In females, after the lower dose it increased by 58 %, and after the highest dose 66 %. In males, the lowest dose increased LH in 1 % and the highest dose by

97 %. Serum collections for LH analysis were performed pre-administration and post-administration at 24 h and 48 h (Karila et al., 1996). Although the LH concentrations increased after both doses of alcohol, these changes did not reach statistical significance ($p < 0.05$).

The analysis of urinary LH concentration was not performed.

4.1.4 Immunoassay

LH is a heterodimeric glycoprotein with each monomeric unit being a glycoprotein molecule comprising of two non-covalently linked subunits, designated α and β. Each monomeric subunit is not biologically active but when combined into the heterodimer the molecule binds to the LH receptor located in the gonads.

The DELFIA® LH immunoassay used for this work is a two-site fluoroimmunometric assay. It consists of two highly specific monoclonal antibodies aimed against two separate epitopes of the LH molecule. An epitope is the antigen's part recognised by the immune system. The standards, samples and quality controls that contain LH are incubated with immobilised monoclonal antibodies which are aimed to a specific antigenic site in the β sub-unit of the LH protein.

The europium-labelled antibodies are targeted at a second site of the β sub-unit and will react with the intact LH or the β sub-unit that has been previously bound to the solid-phase antibody, thus creating a two-site 'sandwich'. The enhancement solution will dissociate the europium from the antibodies it was attached to, forming fluorescent chelates with the enhancement solution. Fluorescence is measured in a time-resolved fluorometer and it is directly proportional to the quantity of LH in the sample.

4.2 Material and methods

4.2.1 Materials

Vacutainers® (SST™II *Advance)* for blood serum separation were obtained from BD Diagnostics (Oxford, UK). The 96-well immunoassay DELFIA® LH kits, the DELFIA® 1296-026 platewasher with a Victor^3 1420 multi-label counter, and the MultiCalc® 2000 software program, were all obtained from PerkinElmer® (Northwhich, UK).

4.2.2 Volunteer study and sample collection

Samples from the volunteer study described in Chapter 3, section 3.2.3 were used. In summary, 9 females and 9 males were recruited and the samples were labelled with an alphanumeric code, e.g. TαF/M-4U-$\underline{\beta\delta}$, where α is the number of the volunteer, β the letter indicating the type of sample (U for urine and S for serum), δ the time of collection and F/M with F if volunteer was a female and M if it was male.

The volunteers were administered with 4 and after with 8 units of alcohol on two different occasions, with at least 2 weeks apart, cannulated and blood was collected pre-alcohol administration, and post-administration hourly from 1 h to 6 h, then at 24 h and at 48 h. The blood was collected into Vacutainers® with a separating gel and allowed to stand for 30 min, after which it was centrifuged for 5 min at 1000 *g*.

Urine was collected pre-administration, and post-administration hourly from 1 h to 7 h, then at 10 h, 24 h, 48 h and 72 h. It was collected in Nalgene® 500 mL bottles, and split into four 10 mL aliquots (when enough urine was available).

The female volunteers were not receiving hormonal contraceptive therapy, and were asked to provide information pertaining to the last day of their period.

4.2.2.1 Serum analysis

The DELFIA® LH immunoassay kit was used as per the protocol provided with the kit, with urinary QCs described below (section 4.2.2.2). This assay has been validated by the

manufacturer according to the FDA guidelines, and the assay's sensitivity is typically better than 0.05 IU/L according to the manufacturer.

4.2.2.2 Urine analysis

The urinary LH method has been validated using a serum DELFIA® LH kit by PerkinElmer®. This validation was performed following the WADA guidelines and it was carried out by certified analysts within the Drug Control Centre. The LOD was determined to be at 0.07 IU/L and the LLOQ was 0.14 IU/L.

The statistical treatment for the volunteer's samples was performed using the software Statistical Package for Social Sciences (SPSS®), version 18, from SPSS® Inc, Chicago, IL, USA. The results were statistically treated by using a general linear model with repeated measures and the significance limit was set to p-value <0.05. This statistical test verifies the effects of alcohol on LH concentration, and by inspection of statistical tables and graphs it is then possible to conclude if any significant changes are due to an increase or a decrease in LH concentration.

4.3 Results and discussion

To adjust for urine volume dilution factors due to the alcohol ingestion, the LH concentrations were calculated taking into account the SG of each sample. Only values normalized for a SG of 1.020 could be compared.

Adjusting urinary concentrations for dilution factors using SG rather than creatinine is common practice especially in anti-doping laboratories. The use of creatinine is more commonly used for adjustments of variable sampling times and/or diurnal rhythm (Miller et al., 2004, Greenberg and Levine, 1989, Handelsman et al., 2009). However, in sport, the use of creatine, which is readily converted to creatinine, is permitted and this makes adjustment to creatinine inappropriate.

Thus, the SG of all urine samples was measured and the LH concentration adjusted according to the following equation:

Equation 4.1 Adjusted concentrations

Concentrations that were below the LLOQ of 0.14 IU/L were adjusted to half of this value for statistical treatment purposes.

Statistical treatment was done by averaging post-administration data irrespective of gender, time or units and normalizing the data using proportion increase against pre-administration as follows:

Equation 4.2 Normalization of values for statistical treatment

During the study, 1 male volunteer withdrew from the study (T3M), and it was not possible to collect blood from 1 female (T3F).

The urinary 72 h sample was excluded from statistical treatment since 1 male and 1 female volunteer failed to provide this sample. The samples collected at 24 h and 48 h had already reached baseline values and a comparison between baseline value (baseline sample taken before alcohol ingestion) and these two samples were done to ascertain if the response was different for the 4 and 8 units. The results were not statistically significant with urinary LH p-value=0.234 and serum LH p-value=0.109 therefore the last two measurements (24 and 48 h) were excluded from the statistical treatment. As an added test of reliability for our baseline values, the mean of pre-administration, 24 h and 48 h samples was used as the baseline value.

The serum LH concentration for females after 4 and after 8 units may be found in Appendix 8.20. The urinary values for both doses may be found in Appendix 8.21. For males, serum concentration after 4 and after 8 units are shown in Appendix 8.22. The urinary LH follows in Appendix 8.23.

The graphs supporting the statistical treatment will be presented as normalized values where the baseline concentration is represented as zero concentration, since our normalization involved proportion increase from the baseline.

A graph with the mean raw values (before statistical treatment) after 8 units, for females and males will be presented as well.

4.3.1 Serum analysis

No female volunteer was receiving hormonal contraceptive (see exclusion criteria) to avoid possible effect on LH (Brun et al., 1987), and they were asked to provide the date of their last menstruation so to ascertain what phase of their menstrual cycle they were in.

Females that had their pre-administration concentrations in the mid-cycle range were excluded from statistical treatment. Their higher values would have interfered with the statistical treatment and thus they were considered as outliers.

Statistical treatment of serum LH values showed no gender difference (p >0.05). There was no response difference for 4 and 8 units (p >0.05) and, although males showed a higher response averaged over time, it remains the same when dose increases (p >0.05). There was no change with time (p >0.05), and the LH concentration time plot for 4 and 8 units (gender averaged) was similar (p >0.05).

Since there were no gender or time effects, the data will be presented in a graph with the mean normalized values for each collection point, gender averaged for the two units as shown in Figure 4.1.

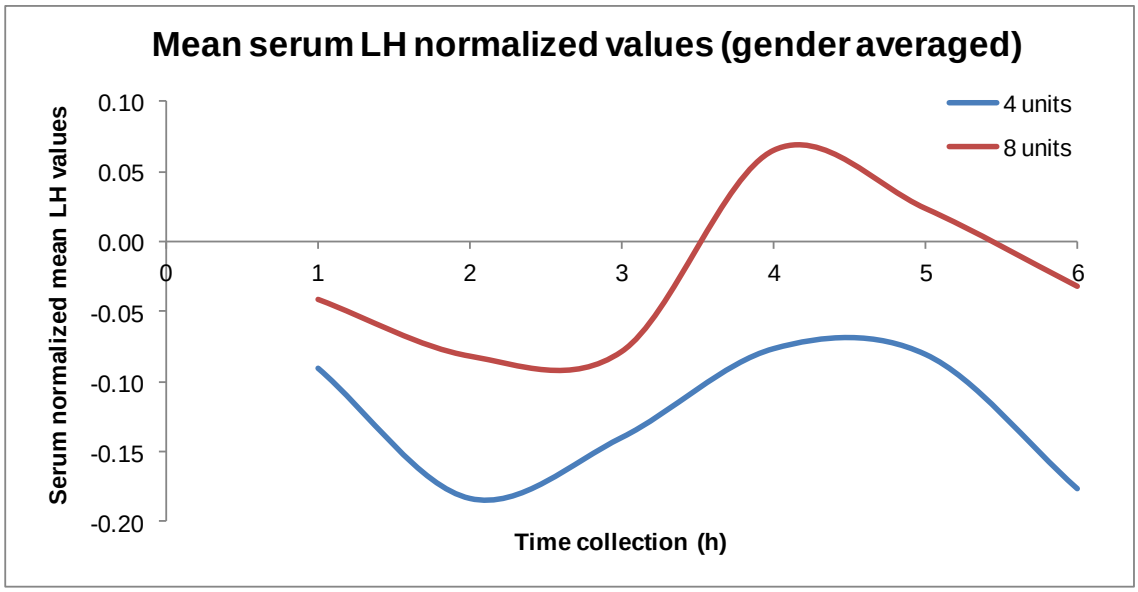


Figure 4.1 LH normalized values in serum, for 4 and 8 units averaged over gender

The graph above shows suppression up to 2 h in both 4 and 8 units, followed by an increase that goes above baseline only after 8 units that starts declining after 4 h, until it is close to baseline.

Although there were no gender differences, the results for the 8 units were split by gender and a graph was plotted accordingly (Figure 4.2).

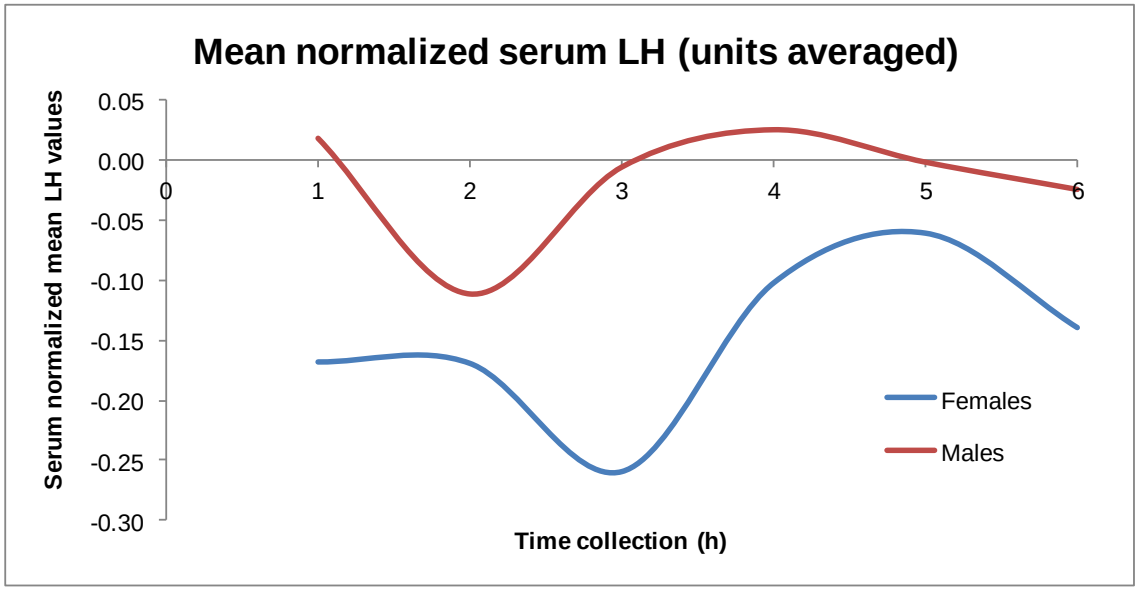


Figure 4.2 LH serum mean normalized concentration for females and males averaged over 4 and 8 units

The statistical results are not significant for females or males, although in males p =0.068 whereas in females the value was 0.518. These results, although intriguing, are not significant.

The measured concentrations before statistical treatment after 8 units of alcohol, for females and males, may be found in Figure 4.3.

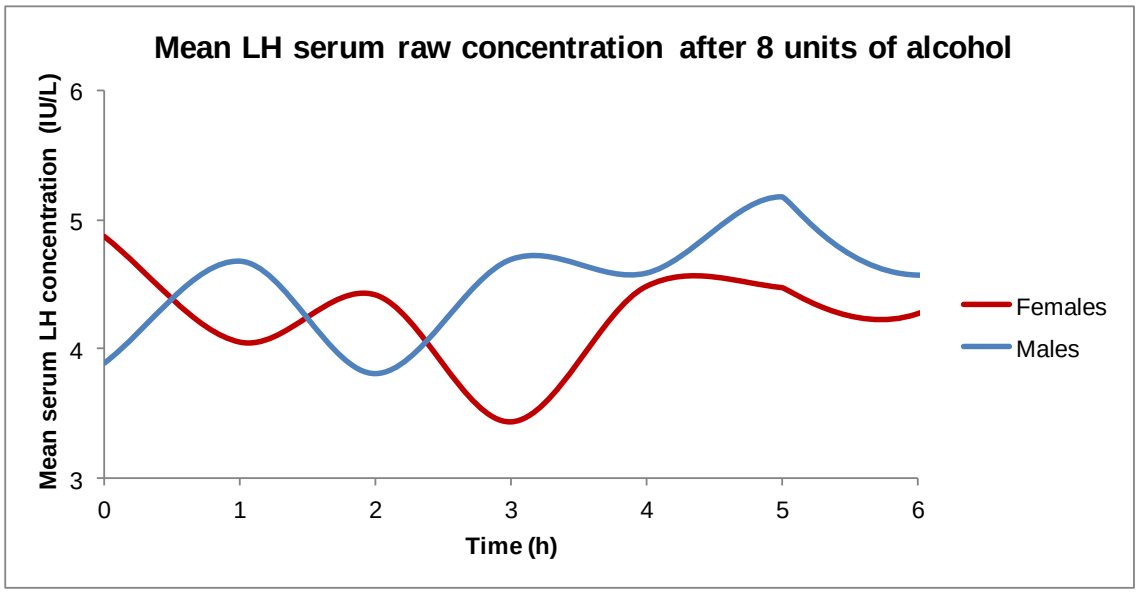


Figure 4.3 LH serum mean concentrations before normalization (raw values), for females and males after 8 units of alcohol

4.3.2 Urine analysis

Urinary LH values were previously adjusted for SG in accordance with Equation 4.1 and graphs were plotted up to 10 h post-administration.

As mentioned previously, there was no statistical difference between pre-administration collection and 24 h and 48 h, therefore the three values were averaged to give a more reliable baseline value. The two female volunteers that were considered outliers were removed from the urinary analysis, and so was one male since his baseline value was above the LH upper concentration limit of 20 IU/L.

The statistical treatment performed in urinary LH also showed no gender difference ($p > 0.05$). There was no response difference for 4 and 8 units ($p > 0.05$), and, although males showed a greater response averaged over time, it remains the same when units increase (p-value > 0.05). There were changes with time (p-value < 0.05), and the LH concentration time plot for 4 and 8 units (male and female averaged) was not significantly different ($p > 0.05$).

Since there was no gender effect, the data will be presented in a graph with the mean values for each collection point, for the different units (Figure 4.4).

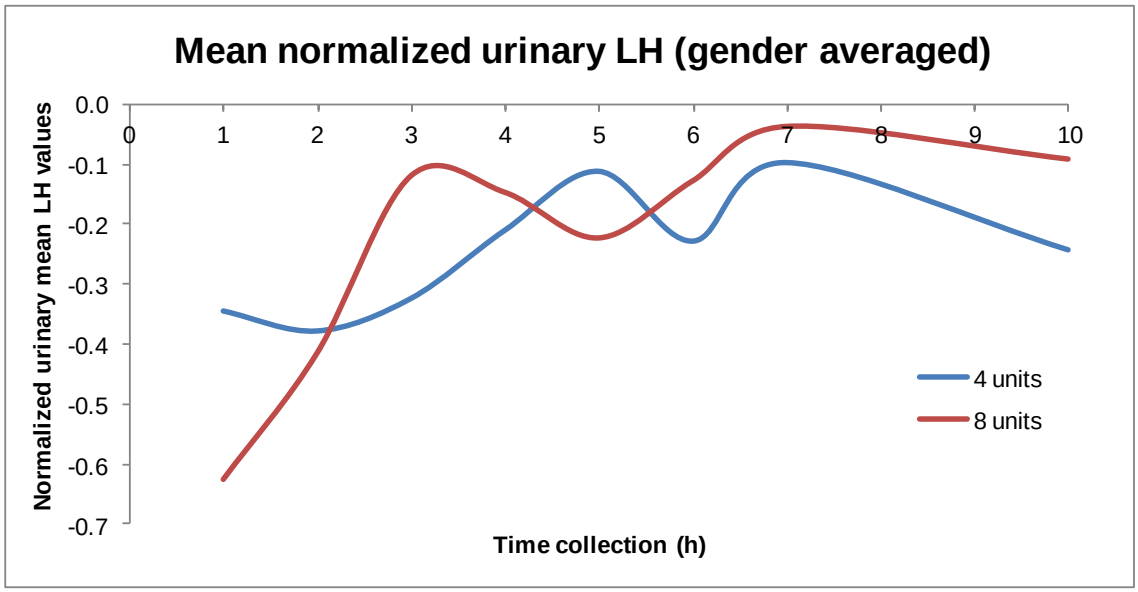


Figure 4.4 Urinary normalized LH mean concentrations, gender averaged, for 4 and 8 units, values previously adjusted for SG

The graph shows suppression up to 2 h after administration of 4 units, followed by an increase up to 5 h that does not reach baseline, it then decreases between 5 h and 6h, increases up to 7 h and decreases again until 10 h, without ever reaching baseline values.

For 8 units, there is higher suppression than for 4 units up to 1 h. It then increases up to 3 h, decreases again until 5 h, increases afterwards until 7 h almost reaching baseline values, and decreases slightly up to 10 h.

Similarly to what happened with serum LH, and although there were no statistically significant differences between both genders, the results from 8 units almost reaching baseline values lead us to analyse the data by splitting the genders. A graph with the mean values may be found below (Figure 4.5).

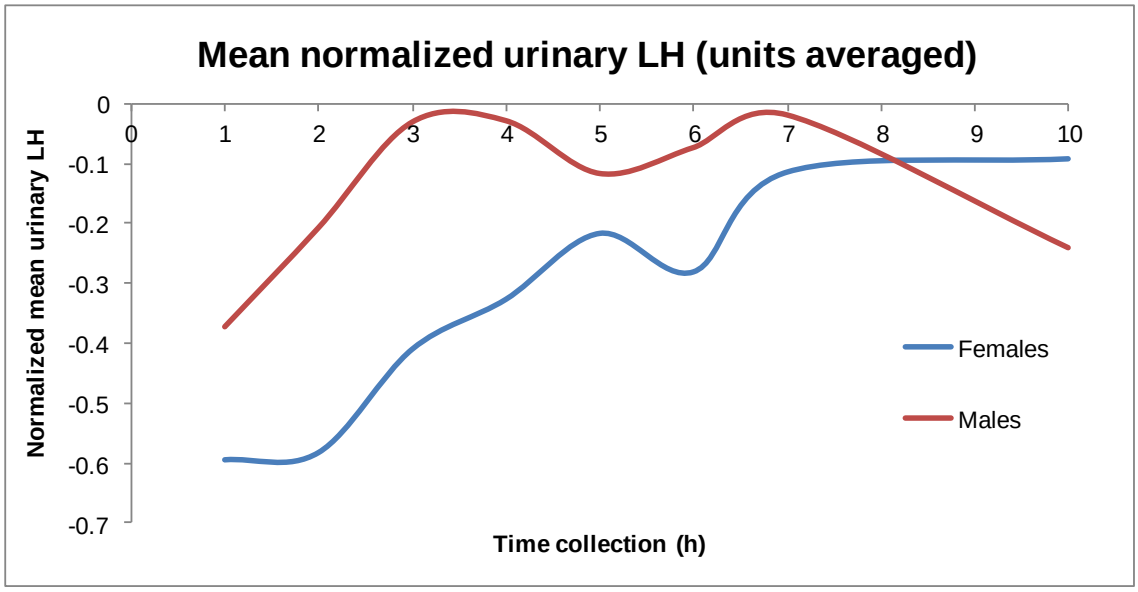


Figure 4.5 LH urinary mean normalized concentrations, for females and males, after 4 and 8 units, values previously adjusted for SG

Although in females there were significant changes over time (p-value < 0.05) with a decrease in LH concentration that was not the case in men as no statistically significant difference for 4 and 8 units was verified.

The data before statistical treatment after 8 units of alcohol, for females and males, will be presented in Figure 4.6.

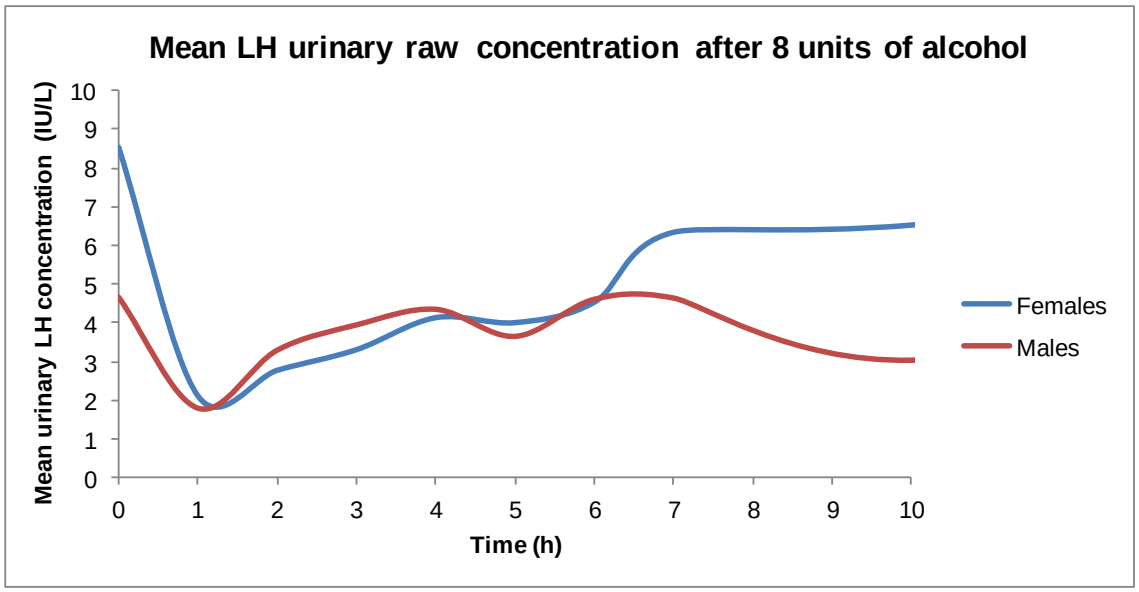


Figure 4.6 LH urinary mean concentrations before normalization, for females and males after 8 units of alcohol

4.3.3 Challenges encountered

Most females were either in their luteal phase or their follicular phase when they took part in this study, which have similar LH concentration. However, female volunteer T5F in the 4 units experiment was in her mid-cycle phase on the day of the study and her LH values were very different from the other volunteers'. Volunteer T4F should have been in the follicular phase of her menstruation although her values are more similar to a mid-cycle phase. We attribute this to possible menstruation irregularities, or having provided the wrong date of her last cycle. These were the volunteers that were considered as outliers.

One male was also removed from the urinary statistical treatment since his pre-administration baseline value was above the 20 IU/L upper limit and it was considered an outlier. This extreme value would distort the data for the remaining subjects.

4.4 Conclusions

Our study shows that 4 and 8 units of alcohol, administered on two different occasions, no change in serum LH concentration in females or males was observed.

This is in accordance with previously published data (Karila et al., 1996, Frias et al., 2002). In the report by Karila *et al.,* the dose administered to 4 females and 4 males was 1.2 g/kg and 2.0 g/kg on two separate occasions for a period of 5 h. LH serum analysis showed an increase in concentration in females by 58 % and 66 %, and in males 1 % and 97 % (lowest and highest dose respectively), with one pre-administration collection and two post-administration collections at 24 h and 48 h. Although mean serum LH increased, these increases were not statistically significant.

In our study, the females showed a decrease in serum LH concentration albeit not statistically significant. With male volunteers, the different units still had a not statistically significant p-value > 0.05, but nevertheless intriguing (p-value = 0.068). It would be interesting to see the effect of alcohol in a bigger population of males.

If a decrease in serum LH concentration was to be verified, it would most likely have to be concordant with an increase in serum T.

Previous studies have shown that administration in men of approximately twice the physiological mean production rate (15 mg/day) of T, reduced LH concentration by 39 ±12 % (Loriaux et al., 1977). More recently, in a study by Geyer *et al*, 100 mg/day of testosterone was administered as a hydroalcoholic gel in eugonadal men (n=9), which is the recommended maximum dose for hypogonadal men on hormone replacement therapy. This dose was administered for 6 weeks, and serum T raised 2 to 3 fold, whereas serum LH decreased to less than 50 % of the pre-administration value in 6 men (Geyer et al., 2007).

Similarly in urine, the administration of 4 and 8 units did not show a statistically significant difference in females and males. After splitting the genders for statistical purposes, averaged over time and units, the females had a statistical significant decrease in the concentrations over time. The athlete defending a doping violation described in the introduction who had a high urinary LH concentration (28.9 IU/L), had a theoretically calculated maximum blood alcohol

concentration of 350 mg%, whereas our highest male volunteer, T10M at 1 h after 8 units, had 111 mg% (Appendix 8.25 for serum alcohol results) and a LH urinary concentration of 1.385 IU/L. Although the alcohol concentration was three times inferior, the urinary LH concentration was twenty times less thus it seems unlikely that his high urinary LH concentration was due to alcohol.

The hypothesis that ethanol administration increases LH concentration thereby increasing urinary T concentration was not confirmed.

The following chapter will verify whether alcohol administration suppresses catabolism of T by enzyme inhibition, e.g. 17β-HSD2, in serum via LC-MS/MS analysis.

Chapter 5 INVESTIGATING THE EFFECT OF ALCOHOL ADMINISTRATION ON SERUM ANDROGENS IN EUGONADAL MEN AND WOMEN

5.1 Introduction

5.1.1 Serum T and alcohol

The target steroids of this chapter were testosterone, epitestosterone, their conjugates (testosterone sulfate, testosterone glucuronide, epitestosterone sulfate and epitestosterone glucuronide), and androstenedione in serum. These steroids are amenable to measurement by LC-MS/MS, necessitating the developement of such an assay for these target analytes. Thus, much of this chapter is devoted to the development and validation of an LC-MS/MS method to quantify these steroids and their conjugates, in serum.

The validated procedure was then employed to investigate whether alcohol administration suppresses catabolism of T by enzyme inhibition, e.g. 17β-HSD2.

Previous studies have demonstrated that alcohol intake (0.5 g/kg) increased serum T in females (Sarkola et al., 2001, Sarkola and Eriksson, 2003, Sarkola et al., 2000). In males, a low dose of alcohol (0.5 g/kg) has been reported to significantly increase serum T (Sarkola and Eriksson, 2003), but with a higher dose (2.0 g/kg) and in very inebriated patients taken to the emergency room the opposite is verified (Frias et al., 2002, Karila et al., 1996).

The proposed pathway to explain the increase in serum T in females and the consequent decrease in AD comes from the suppression of the activity of the 17β-HSD2 enzyme, which is responsible for the catabolism of T into AD. In the oxidation of T to AD by 17β-HSD2, the cofactor NAD^+ is reduced to NADH. The enzymes that oxidise ethanol within the liver are also dependent on the cofactor NAD^+, and Sarkola *et al.* hypothesized that alcohol metabolism is favoured over that of T (Sarkola et al., 2001). Presumably, because the amount of this cofactor presence is limited (Figure 5.1):

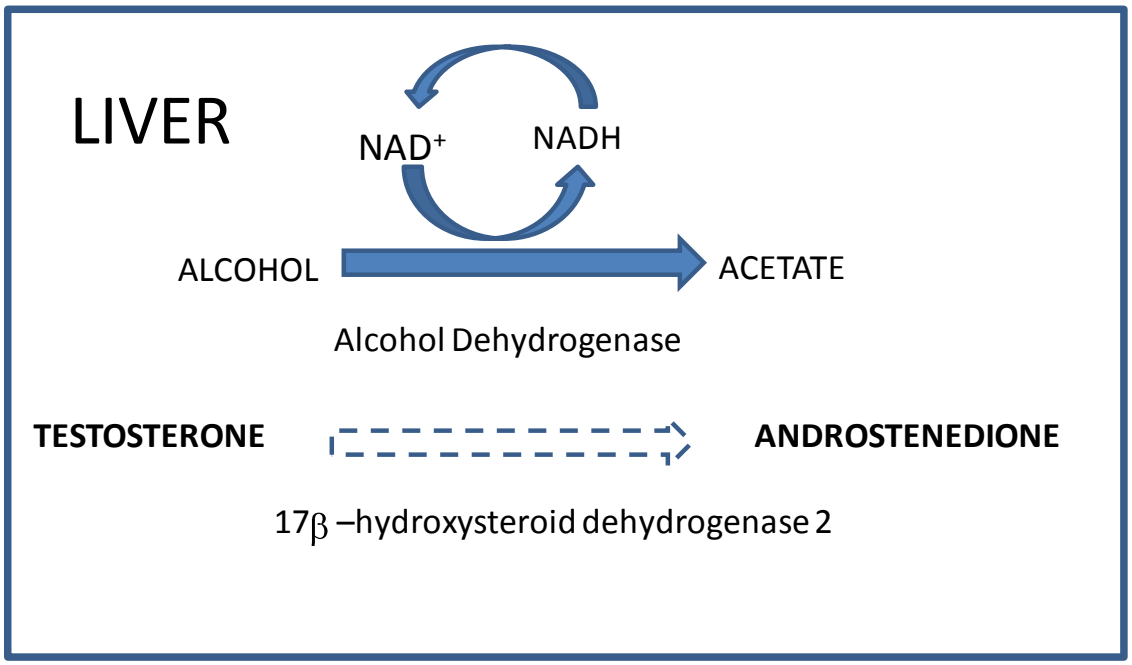


Figure 5.1 Effect of alcohol in the steroid metabolism in the liver, adapted from Sarkola et al. (2001)

Our research studied the effects of alcohol (4 and 8 units on two different occasions) in eugonadal volunteers (9 females and 9 males) on serum testosterone, epitestosterone, their conjugates (testosterone sulfate, testosterone glucuronide, epitestosterone sulfate and epitestosterone glucuronide) and androstenedione.

5.1.2 Liquid-Chromatography Mass- Spectrometry

In liquid chromatography (LC), separation is normally achieved by the partitioning of the solute between the stationary phase and the mobile phase which is a liquid.

Reversed-phase LC stationary phases use non-polar groups (commonly C18) bonded onto silica whereas in capillary GC the stationary phase is normally a non-volatile liquid (i.e. polymethylsiloxane) supported on a capillary column (Harris, 2003).

A detector to register the eluting analytes is connected to the end of the column, such as a MS (Rubinson and Rubinson, 2000b).

The signal produced is typically Gaussian, or bell shaped (Figure 5.2) that describes a random process around the mean of a number of factors such as molecular collision. Chromatographically speaking, the Gaussian peak describes the detector's response (Y) vs retention time (X).

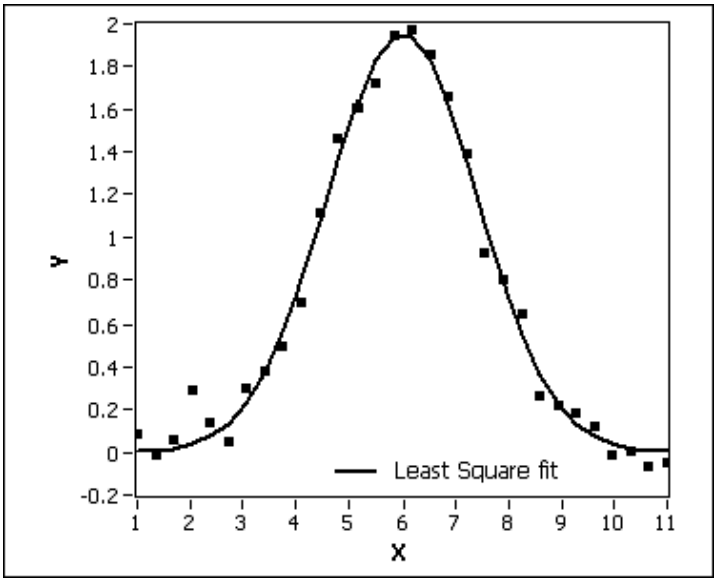


Figure 5.2 Gaussian peak

In LC, the sample is injected into the column and pressure is applied to force the solvent through the column. In reversed-phase chromatography, a polar mobile phase is used and the stationary phase is non polar. The solute can dissolve in the stationary phase, and separation is achieved by a partitioning process based on differences in solute polarity and its solubility.

Although partitioning is the predominant type of separation in this case, there are always other types of interactions such as adsorption and desorption between the solute and the carbon chains of the stationary phase (Christian, 2004b) (Figure 5.3).

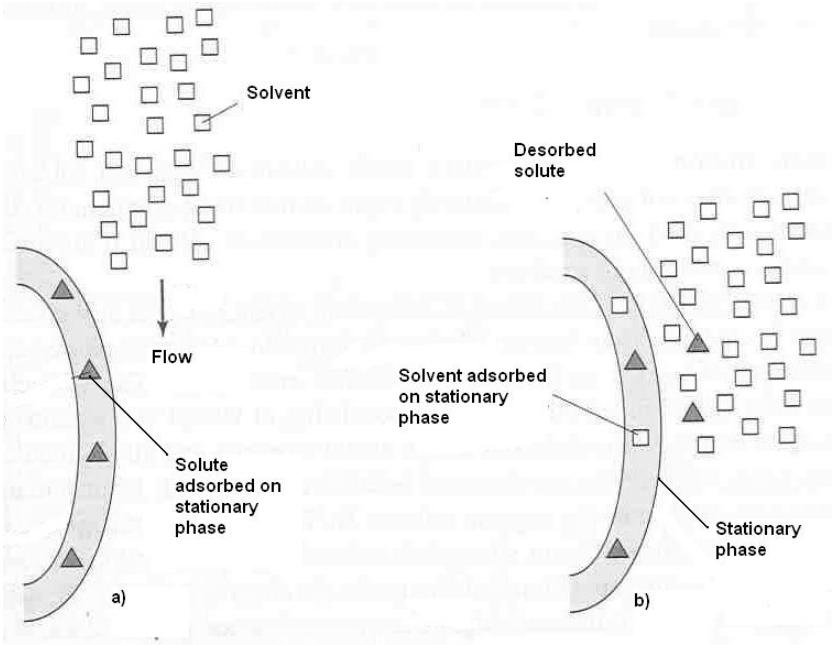


Figure 5.3 Solvent molecules compete with solute ones for binding sites on the stationary phase. a) Solute adsorbed in the stationary phase and b) solvent and solute adsorbed in the stationary phase (Christian, 2004b)

Each column has a limit of the maximum concentration that can be injected, above which partitioning does not occur. Above this concentration, the column is being overloaded as the size of the sample overwhelms the column's capacity to hold the solutes. The 'capacity' of a column is a measure of its ability to bind the amount of material in a sample onto the stationary phase and produce good separation (Harris, 2003, Rubinson and Rubinson, 2000b).

A mass-spectrometer may be coupled to the LC system and used as a detector. The most common ion sources used are electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI), the latter being commonly used for large molecules and non-polar compounds.

The ion source used in this research was ESI and a brief description may be found below. This type of ionisation is good for charged, polar, basic and acidic compounds.

In this ionisation process, the LC eluent containing the analyte is sprayed from a tip to which a strong electric field is applied at atmospheric pressure. The electric field causes a charge accumulation on the liquid surface, forming charged droplets at the tip. These droplets will possess an excess of either positive or negative charge, depending on the polarity of the

capillary. The droplets then pass through a curtain of inert gas at atmospheric pressure or a heated chamber at reduced pressure to evaporate the solvent. After the solvent evaporates, the droplets will reduce to such a small size that ions of the same polarity will repel and generate a Coulombic explosion, causing their division. These droplets undergo a series of explosions, making smaller droplets which pass into the MS detector (Watson and Sparkman, 2007).

The MS used is a triple quadrupole in the selected reaction monitoring (SRM) mode, where only the most intense precursor and product ions are selected to improve sensitivity.

In the first quadrupole (Q1) only the selected precursor ion(s) pass into the second quadrupole (Q2), which acts as a collision cell and fragments the ion(s). Those fragments pass onto the third quadrupole (Q3) where they are separated according to mass to charge and detected (Figure 5.4).

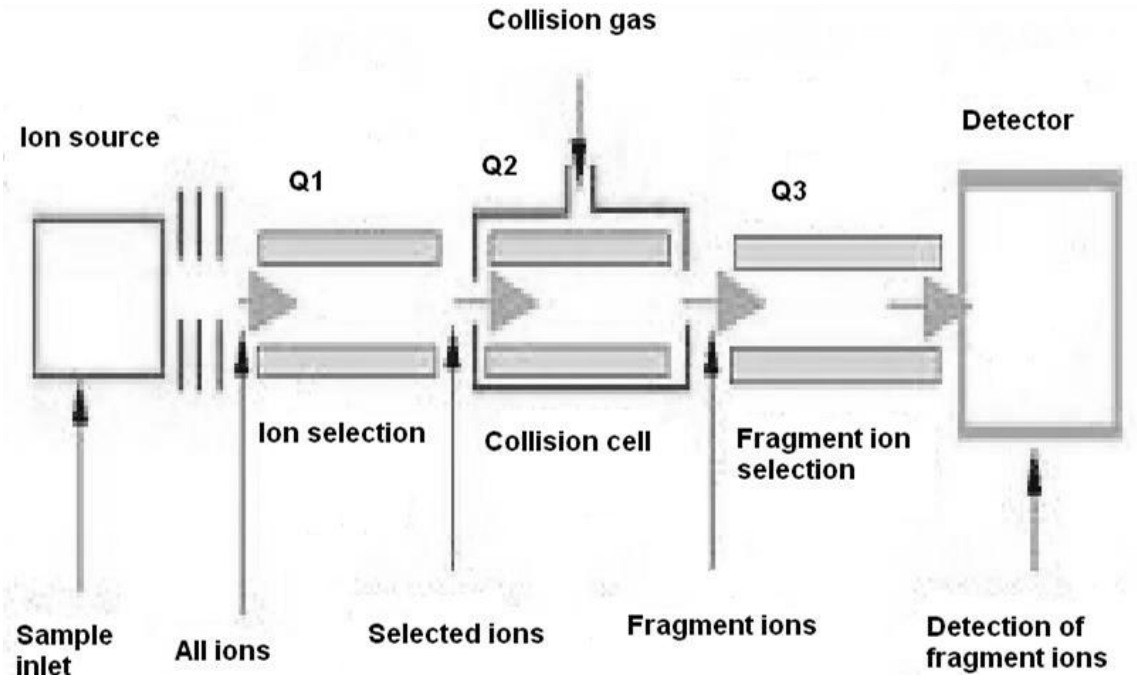


Figure 5.4 Schematic representation of a triple quadrupole mass analyser (adapted) (Scott, 2008)

5.1.3 Analyte concentration range in serum

Before starting the method development and validation, reconnaissance of the literature regarding the concentrations of the analytes of interest in serum was undertaken and the most salient findings displayed in Table 5.1.

Table 5.1 Reference concentrations for free and conjugated steroids of interest in serum in males and females

Steroid	Serum					
	Females			Males		
	nmol/L	ng/mL	Reference	nmol/L	ng/mL	Reference[9]
Androstenedione	1.05 – 6.98	0.3 – 1.9	(National Institute of Health, 2009)	1.4 – 5.2	0.4 - 1.5	(National Institute of Health, 2009)
Epitestosterone	Serum epitestosterone concentration is 15 - 30 times inferior than in males.			0.10 - 1.25	0.03 - 0.361	(Dehennin, 1993)
Epitestosterone glucuronide	There have been no published reports of EG concentrations in blood as concentration is most likely too low.					
Epitestosterone sulphate				0.87 - 4.32	0.32 - 1.59	(Dehennin, 1993)
Testosterone	0.6 - 2.5	0.2 - 0.7	(Burger, 2002)	8.3 - 33	2.4 – 9.5	(National Institute of Health, 2009)
Testosterone glucuronide				1.3 - 2.1	0.60 - 0.97	(Peng et al., 2002)
Testosterone sulphate	N/A[10]	N/A	N/A	0.03 - 14.9 1.1 - 6.3	0.01 - 5.5 0.40 - 2.32	(Dehennin, 1993) (Saez et al., 1967)

[9] Only one reference is displayed for each steroid, with references to earlier relevant publications being therein.
[10] N/A – Not Available. No concentration range was found in the literature.

5.1.4 Method development

Solid-phase extraction (SPE) is commonly used to extract steroids from biological matrices. The extraction procedure used in this research project had been previously developed and used a liquid-liquid extraction (LLE) with ethyl acetate (EtOAc), followed by protein precipitation with acetonitrile and solid-phase extraction (SPE) using 'Hydrophilic Lipophilic Balanced' (HLB) reversed-phase cartridges (Welchman, 2007). For the extraction method, please see section 5.2.4.1.

A literature review performed on steroid analysis by LC-MS showed that LLE using methyl-tertiary-butyl ether (MTBE) or diethyl ether were also used, and were sometimes followed by SPE. A summary of the methods used, as well as the ionisation modes, and LLOQs may be found in Table 5.2. For simplification, steroids that were not of interest were not added to the table.

Table 5.2 Methods for the analysis of nonconjugated and conjugated steroids in serum

Analyte	Gender	Sample preparation	Source and polarity	LLOQ (ng/mL)	Reference
T	M	N/A	APCI	0.2	(Wang et al., 2004a)
T	F & M	LLE (diethyl ether)	APCI, positive mode.	0.09	(Cawood et al., 2005)
T, AD & others	N/A	Protein precipitation	APPI, positive mode	LOD: 0.0015	(Guo et al., 2006)
T	F	LLE (MTBE) & SPE	APCI, positive mode	0.01	(Kushnir et al., 2006)
AD & T	F & M	LLE (MTBE)	ESI, positive mode	0.07	(Gallagher et al., 2007)
T, TG, TS, E, EG, ES	M	LLE (EtOAc) & SPE	ESI, positive mode	0.35 (T), 0.14 (E), 0.2 (TG), 0.5 (EG), 0.2 (TS) 0.4 (ES)	(Welchman, 2007)
T & others	M	MTBE & SPE	ESI, negative mode	0.05	(Licea-Perez et al., 2008)
T & others	F & M	LLE (hexane:ethyl acetate 3:1)	APPI, positive mode	0.01	(Harwood and Handelsman, 2009)
T & others	M	LLE (diethyl ether) SPE	ESI source, positive mode	0.09	(Yamashita et al., 2009)

5.1.5 Choice of calibrants and quality controls

The choice of calibrants prepared took into account the reference range for eugonadal men and women, and also that sometimes the upper limit may be exceeded (in volunteers receiving T replacement therapy; (Chapter 6). (Electronic Medicines Compendium, 2011).

The minimum number of calibrants used was 6 but, in some cases, a higher number of standards were employed to cover for both female and male reference ranges. With T, the range was split in two to cover both ranges with calibrant 6 being the overlapping point.

Table 5.3 Calibrant concentration in ng/mL

Steroid	1	2	3	4	5	6	7	8	9	10	11
Testosterone	0.05	0.08	0.1	0.5	1	2.5	5	7.5	10	15	20
Epitestosterone	0.05	0.08	0.1	0.25	0.5	0.75	-	-	-	-	-
Androstenedione	0.25	0.5	0.75	1	1.5	2	-	-	-	-	-
TG	0.05	0.1	0.25	0.5	0.75	1	1.5	2	-	-	-
TS	0.25	0.5	0.75	1	1.5	2	5	7.5	-	-	-
ES	0.5	0.75	1	1.5	2	2.5				-	-
EG	0.5	0.75	1	1.5	2	2.5				-	-

For the quality controls, serum from female and male volunteers was collected and steroid concentration measured. As mentioned in Chapter 3, the criteria for a low QC is to be ideally around 25 % of the calibration range, the medium QC in the middle range, and the high QC between 75-100 % of the highest concentration in the range. A low, medium and high QC was prepared for all the steroids, except for T where a low and high for each range was decided.

The serum was spiked to achieve the appropriate QC concentration, which may be found in Table 5.4.

Table 5.4 QC range for serum analysis in LC-MS/MS (ng/mL)

Steroid	Low 1	High 1	Low 2	High 2
Testosterone	0.06	1.25	3.00	17.00
	Low	**Medium**	**High**	
Epitestosterone	0.08	0.40	0.70	
Androstenedione	0.27	1.30	1.80	
TG	0.12	0.60	1.25	
EG	0.60	1.25	2.00	
TS	0.25	1.00	6.50	
ES	0.60	1.00	2.00	

5.1.6 Method validation

The method was validated against accepted criteria (CPMP/ICH/281 & 381 guidelines) for linearity, specificity, lower limit of quantification (LLOQ), accuracy and precision. These were the same parameters as were used for validating the method in Chapter 3, section 3.1.2.

5.2 Material and methods

5.2.1 Materials

Vacutainers® (SST™II *Advance)* for blood serum separation were obtained from BD Diagnostics (Oxford, UK). T, E, AD, TG, EG, TS and ES were obtained from Promochem (Teddington, UK). Ammonium acetate, ethyl acetate, acetonitrile and methanol (both HPLC grade) were all obtained from Fisher Scientific (Loughborough, UK). The deuterated internal standards d_3T, d_3E, d_3AD, d_3TG, d_3EG, d_3TS and d_3ES all labeled at positions 16, 16, 17α except for AD which is at position 19, were supplied by the National Analytical Reference Laboratory (Sidney, Australia). The Oasis® HLB cartridges were obtained from Waters™ (Manchester, UK). The centrifuge tubes were purchased from Eppendorf™ (Cambridge, UK). PBS tablets and human albumin solution from Sigma-Aldrich (Poole, UK).

5.2.2 Volunteer study and sample collection

The serum samples used were those collected from the volunteer study described in Chapter 3, section 3.2.3. In summary, 9 females and 9 males were recruited and their samples labelled accordingly. The volunteers were administered with 4 or 8 units of alcohol at least 2 weeks later, cannulated and blood was collected pre-alcohol administration, and post-administration hourly from 1 h to 6 h, then at 24 h and at 48 h. The blood was collected into Vacutainers® with a separating gel and allowed to stand for 30 min, after which it was centrifuged for 5 min at 1,000 *g*.

The female volunteers were not on any hormonal contraceptive.

The alcohol concentration in serum was measured by Mr. Peter Streete from the Medical Toxicology Laboratory at St. Thomas' Hospital, GSTS Pathology (see Appendix 8.24, Appendix 8.25, Appendix 8.26 and Appendix 8.27).

5.2.3 Preparation of calibrant standards and deuterated mixture

5.2.3.1 Combined internal standard mixture

To prepare the stock solution, the ampoules containing the liquid internal standard of interest at approximately 1 mg (d_3T, d_3E, d_3AD, d_3TG, d_3EG, d_3TS and d_3ES) was opened and its contents dissolved in methanol and transferred into a 10 mL volumetric flask. The ampoule was washed with methanol approximately 10 times. The solutions were kept in the freezer at an indicated temperature -20 °C. The final internal standard mixture was prepared in 50/50 CH_3OH/H_2O (200 mL) at the concentrations shown in Appendix 8.29. To each calibrant and sample, 25 μL of the mixture was added and the final concentration present in each sample shown in Appendix 8.28 and Appendix 8.29.

5.2.3.2 Artificial serum

Artificial serum (Raggi et al., 1999) was prepared by spiking PBS (0.05 M) with human serum albumin (0.56 mM, 3.36 g in 10 mL) to better mimic the concentration found in human serum (Dunn et al., 1981).

5.2.3.3 Preparation of calibrants

All the calibrants were prepared in artificial serum. To 490 μL of artificial serum, 10 μL of previously prepared calibrants (in water) were added and spiked with 25 μL of the internal standard mixture.

5.2.4 Experimental design

5.2.4.1 Extraction procedure

For the extraction, 500 μL of sample were transferred into 2 mL centrifuge tubes together with 25 μL of internal standard and 1 mL of ethyl acetate. The samples were vortex mixed then centrifuged for 5 min at 17,320 *g*. The supernant was transfered into a glass tube, and to the aqueous layer (serum) 1 mL of acetonitrile was added. The samples were vortex mixed and

centrifuged again as described previously. The supernant was transferred to the glass tube and the samples evaporated under nitrogen at 80°C ±5 °C for 20 min. The samples were then reconstituted in 500 μL of PBS for loading onto SPE cartridges. The HLB cartridges were conditioned with methanol (1 mL) and equilibrated with water (1 mL). The samples (500 μL) were then loaded onto the cartridge, washed with water (1 mL). Elution was with ethyl acetate (1 mL) and then methanol (1 mL), the organic layer solvent was then evaporated at 80°C ±5 °C under nitrogen for 15 min.

The samples were reconstituted in 100 μL of LC mobile phase and centrifuged under the same conditions as above. The supernants were transfered into autosampler vials and analysed by LC-MS/MS.

5.2.4.2 LC-MS/MS conditions

The LC-MS/MS method was developed and validated using a Waters™ XEVO TQS, (triple quadrupole) with an ESI source. The LC column used was a Waters™ C_{18} BEH phase, 2.1 x 50 mm, 1.7 μm particle size. The column temperature was kept at 30°C and the mobile phase was composed of 7.5 mM of ammonium acetate in water with 0.1 % acetic acid (A), and 7.5 mM of ammonium acetate in methanol with 0.1 % acetic acid (B).

The elution conditions were as provided in Table 5.5.

Table 5.5 Elution gradient in LC-MS/MS method

Time (min)	Mobile phase (A/B)
0.5	60/40
3	40/60
4	25/75
4.1	10/90
5	10/90
6	60/40

5.2.5 Data analysis

The statistical treatment in data analysis was performed using the software Statistical Package for Social Sciences (SPSS®), version 18, from SPSS® Inc, Chicago, IL, USA. The results were statistically treated using a general linear model with repeated measures. The significance limit was set to p-value <0.05. This statistical treatment tests for significant changes and by inspection of graphs and statistical tables it is possible to say if that change is an increase or a decrease.

All the calibrants and samples were plotted using a linear weighted regression calibration. Integration of the peak area was performed by using the software TargetLynx® version 4.1 Waters®.

5.3 Results and discussion

5.3.1 Method validation

The transitions for the analytes were determined by infusion. They were detected and quantified in selected reaction monitoring (SRM) mode and the transitions monitored for each analyte were the most intense ones shown in Table 5.6. The table shows the precursor, the quantifier and the qualifier ions, together with the transition's collision energy and cone voltages. The integration period time for each peak was set to a few seconds before/after the peak started/finished, which was at approximately 0.20 min of the retention of the time of each peak's maximum.

Table 5.6 SRM transitions of steroids of interest in LC-MS/MS

Steroid	Precursor (m/z)	Quantifier ion (m/z)	Qualifier ions (m/z)	Cone Voltage (V)	Collision Energy (V) of quantifier ion
T	289	97	97, 109, 123	25	20
d_3T	292	97	97, 109	25	20
E	289	97	97, 109, 123	38	22
d_3E	292	97	97, 109, 274	25	22
AD	287	97	97, 109, 123	20	20
d_3AD	290	100	100, 109	25	20
TG	465	289	289, 109, 253	34	18
d_3TG	468	97	97, 292, 109	25	25
EG	465	289	289, 271, 109	25	10
d_3EG	468	97	97, 109	25	30
TS	369	97	97, 109, 123	15	22
d_3TS	373	97	97, 109	25	22
ES	369	271	271, 97	15	15
d_3ES	373	97	97, 109	25	24

The capillary voltage was optimized for T and set at 1 kV. The desolvation temperature was 500 °C and the gas flow 1,000 L/hr.

With the current 6 min method, all the analytes were totally resolved (see Figure 5.5 showing the product ion chromatogram from the High QC). Only the first 4 minutes of the run is displayed, as all the analytes had eluted within that time.

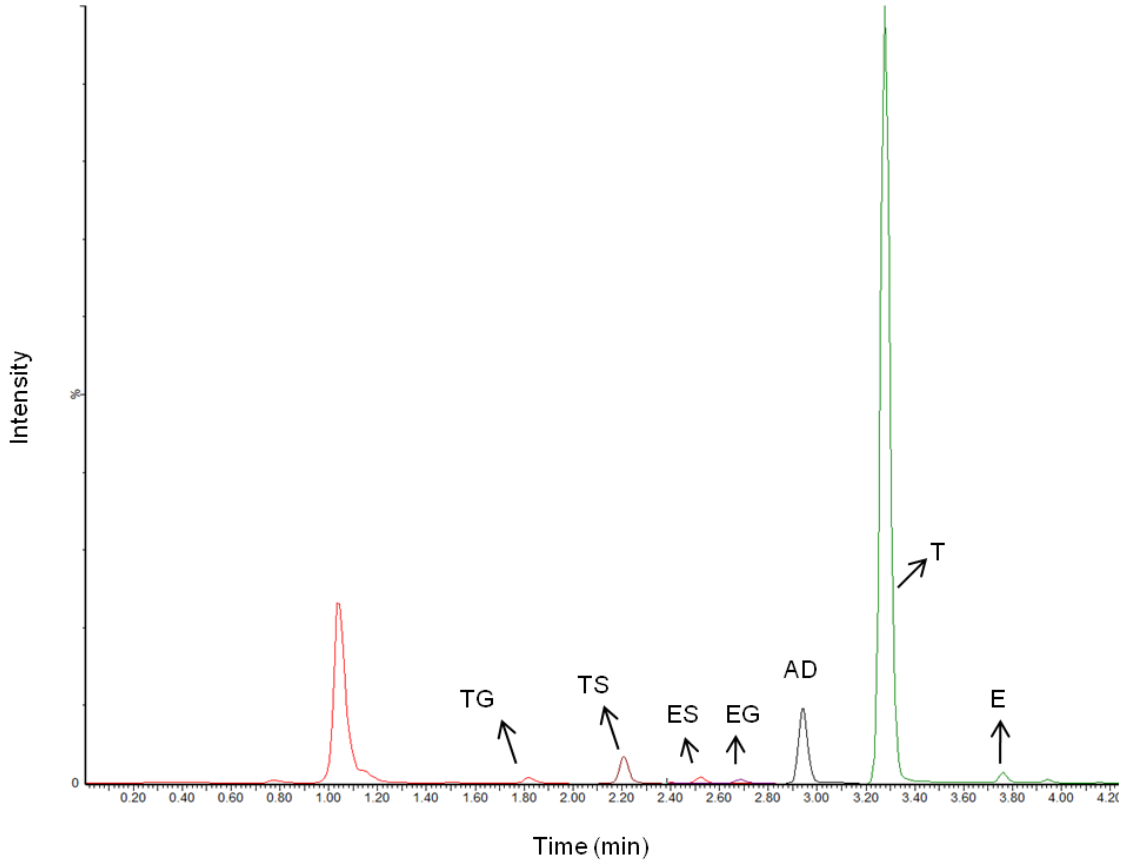


Figure 5.5 LC-MS/MS chromatogram of the high QC with the 7 analytes monitored: TG, TS, ES, EG, AD, T and E (ng/mL)

To assure that in the serum samples to be analysed there would be no co-eluting analytes that would have the same retention time and transitions as our compounds, a long 18 minute-run method was performed to check that no co-eluting compounds were interfering in the 6 minute run.

Both positive and negative mode were tried, and although the sulphates showed more intense peak transitions in negative mode, ES co-eluted with dehydroepiandrosterone (DHEA) sulphate. This compound has the same molecular weight and SRM transitions as ES and it is present in serum at concentrations between 800-5,600 ng/mL in males and 350-4,300 ng/mL in females (National Institute of Health, 2009), therefore preventing the detection and quantification of ES. DHEA sulphate was not detected in positive mode thus this was the mode chosen for

measuring ES, which does ionise in both negative and positive modes (although more sensitive in the former).

5.3.1.1 Calibrant matrix

A suitable matrix for calibrants was sought in order to be able to have good traceability of analyte concentration while reliably mimicking blood serum, the matrix of interest.

Calf serum was analysed but found to contain the steroids being targetted and thus was excluded as a matrix.

Steroid stripped serum was also investigated but also contained small amounts of target steroids and therefore a laborious method to strip the serum would have been required.

Artificial serum was prepared (see section 5.2.3.2), spiked with 5 ng/mL of T (methanolic solution added with a glass syringe) and extracted in triplicate. This concentration was compared with a serum sample containing the same concentration (estimated by standard addition).

Standard addition is a method in which the exact concentration of a sample is determined by adding constant increments of the analyte of interest. In this case, 5 ng/mL of testosterone (equivalent to the approximate concentration estimated to be present) were added, and, from the resulting line, the intercept calculated to determine the concentration in the original sample.

A two-sample *t*-test assuming equal variance was performed on the concentrations of obtained from artificial serum and human serum. The variances were not statistically different ($p > 0.05$), strongly indicating that there was no difference in matrix effects regarding the measurement of T (and most likely the other target analytes). The artificial serum was, therefore, deemed suitable as a matrix for preparing the calibrants.

5.3.1.2 LLOQ

The LLOQ was measured using the smallest concentration of analyte that can be measured for which the S/N is 1:10 prepared in artificial serum. The concentration of the smallest analyte

should have an accuracy and precision of < 20 % and therefore each standard whose initial estimate of S/N was 10:1, was prepared and extracted 4 times.

The LLOQs for T, E and TG were 0.05 ng/mL, 0.25 ng/mL for AD and TS and 0.5 ng/mL for EG and ES.

5.3.1.3 Extraction recovery

The extraction recovery was determined by comparing the signals (peak area) from analysis of extracted calibrants with un-extracted standards, in the low, medium and high range for T and in the low and high range for AD, the main analytes of interest. For T, at 0.5 ng/mL recovery was 64 %, at 5 ng/mL 103 % and at 15 ng/mL it was 124 %. For AD, at 0.5 ng/mL it was 113 % and at 2 ng/mL it was 90 %.

5.3.1.4 Carry-over

Carry-over was tested by injecting a five times a mobile phase blank sample after the highest calibrant standard. The presence of any of the analytes was < 0.1 % of peak area.

5.3.2 Validation criteria

Validation was performed by extracting each QC three times on three different days. The concentration of each QC was calculated by plotting it against a calibration curve with standards prepared fresh on the day. The parameters used were the same ones as mentioned in Chapter 3 which may be found below:

Equation 5.1 Accuracy (within assay)

$$\frac{\qquad\qquad}{\qquad\qquad}$$

(< ± 20 % LLOQ, < ± 15 % other points)

Equation 5.2 Average accuracy (between assay)

(< ± 10 %)

Equation 5.3 Repeatability

(< 10 %)

Equation 5.4 Reproducibility

(< 10 %)

Equation 5.5 Average precision (between assay)

(< 10 %)

The QCs were monitored in every run and they were within the acceptance criteria of ±2 SD). This represents 20 % of the relative standard deviation.

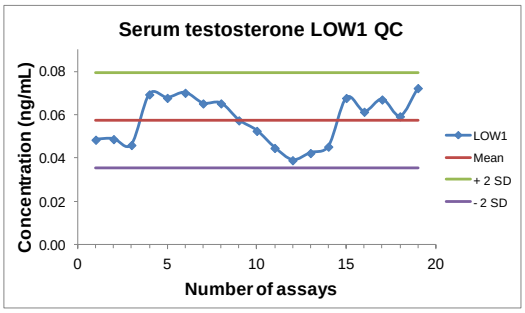


Figure 5.6 LOW1 QC monitoring for serum T (low range)

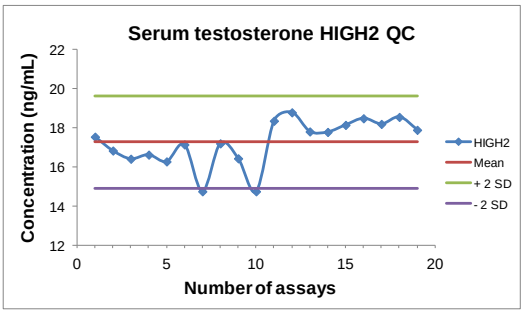


Figure 5.9 HIGH2 QC monitoring for serum T (high range)

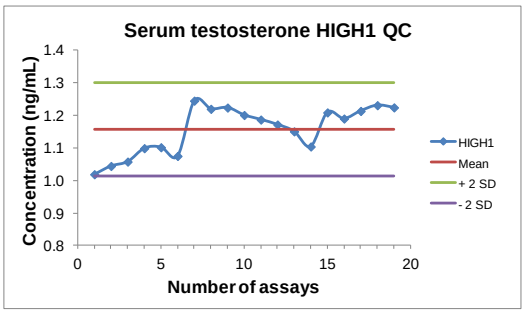


Figure 5.7 HIGH1 QC monitoring for serum T (low range)

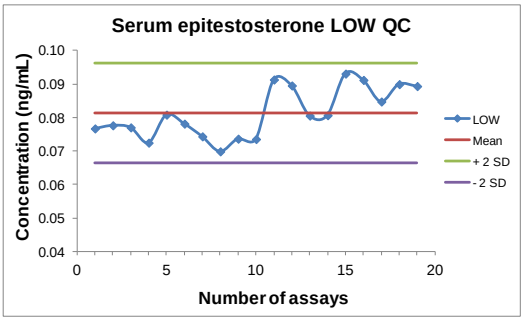


Figure 5.10 LOW QC monitoring for serum E

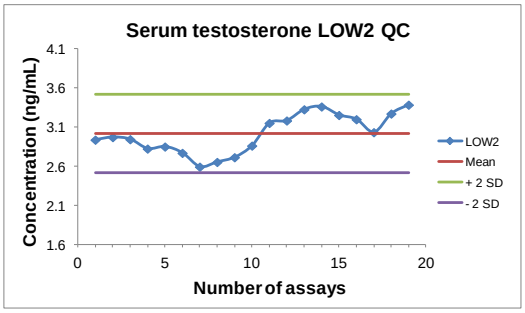


Figure 5.8 LOW2 QC monitoring for serum T (high range)

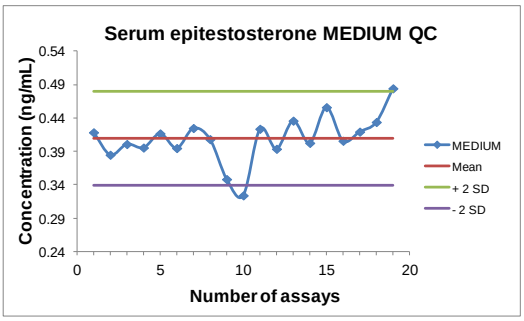


Figure 5.11 MEDIUM QC monitoring for serum E

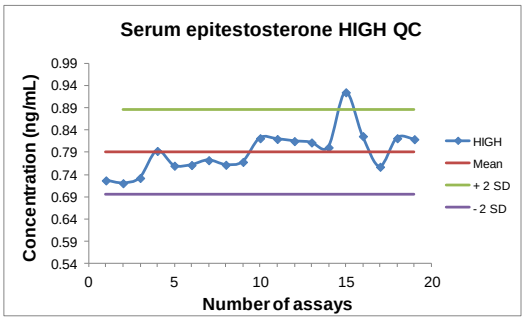


Figure 5.12 HIGH QC monitoring for serum E

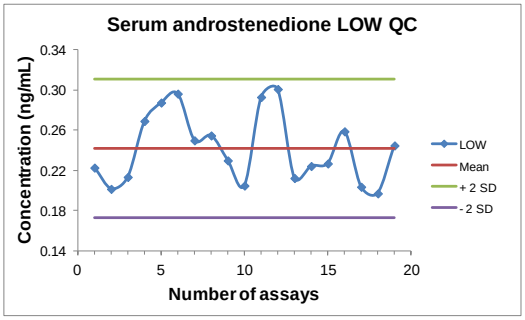


Figure 5.13 LOW QC monitoring for serum AD

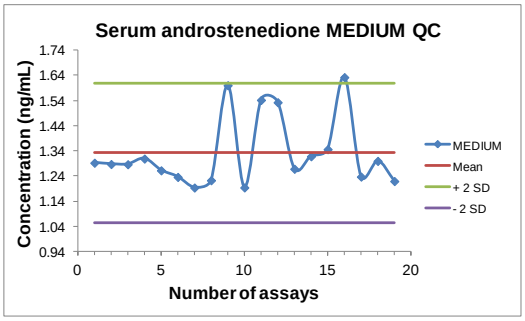


Figure 5.14 MEDIUM QC monitoring for serum AD

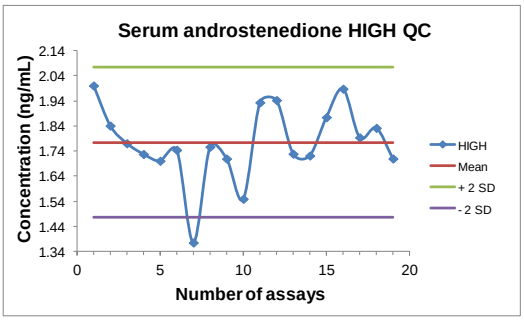


Figure 5.15 HIGH QC monitoring for serum AD

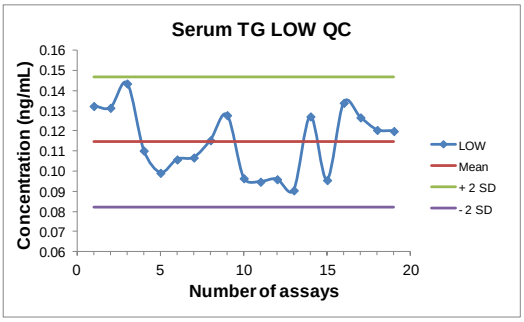


Figure 5.16 LOW QC monitoring for serum TG

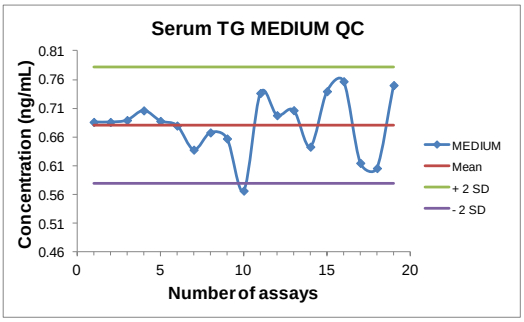


Figure 5.17 MEDIUM QC monitoring for serum TG

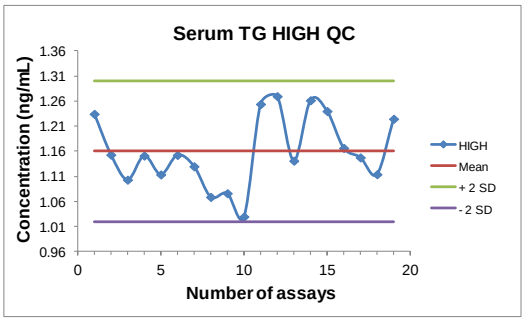


Figure 5.18 HIGH QC monitoring for serum TG

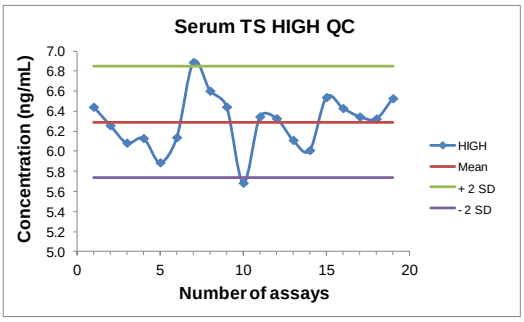


Figure 5.21 HIGH QC monitoring for serum TS

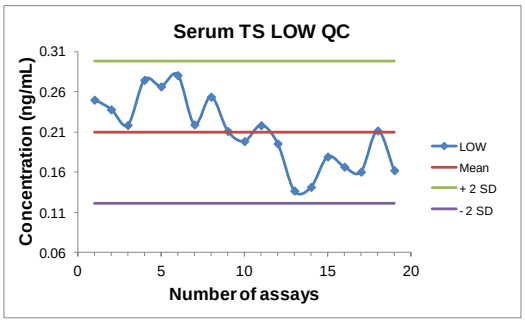


Figure 5.19 LOW QC monitoring for serum TS

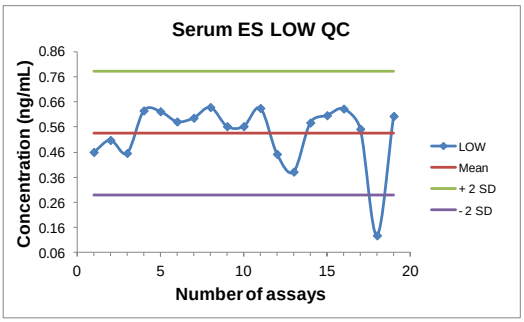


Figure 5.22 LOW QC monitoring for serum ES

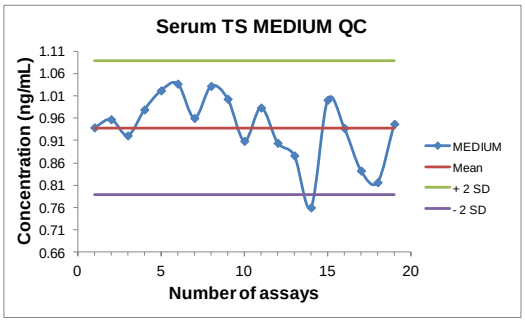


Figure 5.20 MEDIUM QC monitoring for serum TS

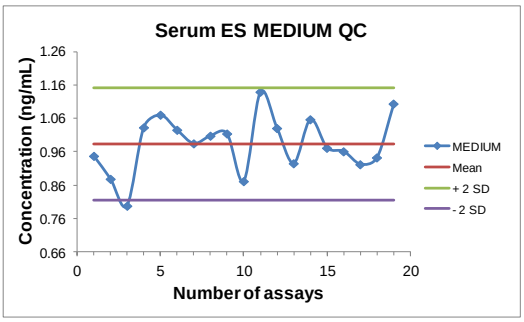


Figure 5.23 MEDIUM QC monitoring for serum ES

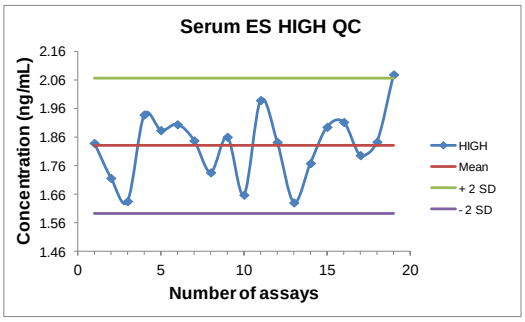


Figure 5.24 HIGH QC monitoring for serum ES

5.3.3 Method validation for the quantification of serum T

5.3.3.1 Calibration range

The lowest calibrant, at the LLOQ, had a percentage accuracy (within assay) <±20%, and the remaining calibrants <±15 %, passing the validation criteria. The linearity of the calibration plots (r^2 value), for the 3 day validation of T, for both ranges was 0.99.

5.3.3.2 Quality controls validation

Table 5.7 Validation of serum T for LOW1 and HIGH1 QCs (low range)

	Day 1	Day 2	Day 3
LOW1 QC (ng/mL)	0.05	0.07	0.07
	0.05	0.07	0.07
	0.05	0.07	0.06
Mean (ng/mL)	0.05	0.07	0.06
% Accuracy	-20.3	15.3	4.5
SD (ng/mL)	0.0	0.0	0.0
% CV	3.1	1.7	7.2

	Day 1	Day 2	Day 3
HIGH1 QC (ng/mL)	1.02	1.10	1.25
	1.05	1.10	1.22
	1.06	1.08	1.22
Mean (ng/mL)	1.0	1.1	1.2
% Accuracy	-16.7	-12.6	-1.6
SD (ng/mL)	0.0	0.0	0.0
% CV	1.9	1.3	1.1

Table 5.8 Validation parameters of serum T for LOW1 QC and HIGH1 QC (low range)

LOW1 QC	
%Accuracy (between assay)	-0.2
%Precision (within assay)	4.0
%Precision (between assay)	4.0

HIGH1 QC	
%Accuracy (between assay)	-10.3
%Precision (within assay)	1.4
%Precision (between assay)	1.4

Table 5.9 Validation of serum T for LOW2 and HIGH2 QCs (high range)

	Day 1	Day 2	Day 3
LOW2 QC (ng/mL)	2.94	2.83	2.60
	2.97	2.85	2.66
	2.95	2.77	2.72
Mean (ng/mL)	2.95	2.82	2.66
% Accuracy	-1.5	-6.0	-11.4
SD (ng/mL)	0.0	0.0	0.1
% CV	1	1	2

	Day 1	Day 2	Day 3
HIGH2 QC (ng/mL)	17.56	16.64	14.77
	16.85	16.30	17.22
	16.43	17.16	16.45
Mean (ng/mL)	16.9	16.7	16.1
% Accuracy	-0.3	-1.8	-5.0
SD (ng/mL)	0.6	0.4	1.3
% CV	3.4	2.6	7.8

Table 5.10 Validation parameters for serum T's LOW2 QC and HIGH2 QC

LOW2 QC	
%Accuracy (between assay)	-6.3
%Precision (within assay)	1.4
%Precision (between assay)	1.4

HIGH2 QC	
%Accuracy (between assay)	-2.4
%Precision (within assay)	4.6
%Precision (between assay)	4.5

The accuracy should be < ±15 % or < ±20 % if it is at the LLOQ and the CV < ±10 %. All the QCs in the low range and in the high range passed the criteria so the method was successfully validated for serum T.

5.3.4 Method validation for the quantification of serum E

5.3.4.1 Calibration range

The lowest calibrant, at the LLOQ, had a percentage accuracy (within assay) <±20%, and the remaining calibrants <±15 %. The linearity of the calibration plots (r^2 value), for the 3 day validation of E, from 0.05 to 0.75 ng/mL was 0.99.

5.3.4.2 Quality controls validation

Table 5.11 Validation of serum E for LOW, MEDIUM and HIGH QCs

	Day 1	Day 2	Day 3
LOW QC (ng/mL)	0.08	0.07	0.07
	0.08	0.08	0.07
	0.08	0.08	0.07
Mean (ng/mL)	0.08	0.08	0.07
% Accuracy	-3.5	-3.5	-9.1
SD (ng/mL)	0.0	0.0	0.0
% CV	0.6	5.5	3.3

	Day 1	Day 2	Day 3
MEDIUM QC (ng/mL)	0.42	0.40	0.43
	0.39	0.42	0.41
	0.40	0.40	0.35
Mean (ng/mL)	0.40	0.40	0.39
% Accuracy	0.4	0.7	-1.5
SD (ng/mL)	0.0	0.0	0.0
% CV	4.2	3.1	10.2

	Day 1	Day 2	Day 3
HIGH QC (ng/mL)	0.73	0.79	0.77
	0.72	0.76	0.76
	0.73	0.76	0.77
Mean (ng/mL)	0.73	0.77	0.77
% Accuracy	3.9	10.3	9.7
SD (ng/mL)	0.0	0.0	0.0
% CV	0.8	2.4	0.7

Table 5.12 Validation parameters of serum E for LOW, MEDIUM and HIGH QC

LOW QC	
%Accuracy (between assay)	-5.4
%Precision (within assay)	3.1
%Precision (between assay)	3.1

MEDIUM QC	
%Accuracy (between assay)	-0.1
%Precision (within assay)	5.8
%Precision (between assay)	5.8

HIGH QC	
%Accuracy (between assay)	8.0
%Precision (within assay)	1.3
%Precision (between assay)	1.3

The accuracy and CV were below the required parameters, and the calibrants were within the acceptance criteria thus the QCs passed the criteria and the method was validated for serum E.

5.3.5 Method validation for the quantification of serum AD

5.3.5.1 Calibration range

The lowest calibrant, at the LLOQ, had a percentage accuracy (within assay) <±20%, and the remaining calibrants <±15 %. The linearity of the calibration plots (r^2 value), for the 3 day validation of AD, from 0.25 to 2 ng/mL was 0.99.

5.3.5.2 Quality controls validation

Table 5.13 Validation of serum AD for LOW, MEDIUM and HIGH QCs

	Day 1	Day 2	Day 3
LOW QC (ng/mL)	0.24	0.27	0.25
	0.22	0.29	0.26
	0.23	0.30	0.23
Mean (ng/mL)	0.23	0.28	0.25
% Accuracy	-16.2	5.4	-9.2
SD (ng/mL)	0.0	0.0	0.0
% CV	4.6	4.9	5.4

	Day 1	Day 2	Day 3
MEDIUM QC (ng/mL)	1.29	1.31	1.19
	1.29	1.26	1.22
	1.29	1.24	1.60
Mean (ng/mL)	1.29	1.27	1.34
% Accuracy	-0.8	-2.3	3.1
SD (ng/mL)	0.0	0.0	0.2
% CV	0.3	2.9	16.9

	Day 1	Day 2	Day 3
HIGH QC (ng/mL)	2.00	1.73	1.38
	1.84	1.70	1.76
	1.77	1.75	1.71
Mean (ng/mL)	1.87	1.73	1.62
% Accuracy	3.7	-4.1	-10.3
SD (ng/mL)	0.1	0.0	0.2
% CV	6.3	1.3	12.9

Table 5.14 Validation parameters of serum AD for LOW, MEDIUM and HIGH QC

LOW QC	
%Accuracy (between assay)	-6.7
%Precision (within assay)	5.0
%Precision (between assay)	5.0

MEDIUM QC	
%Accuracy (between assay)	0.0
%Precision (within assay)	6.7
%Precision (between assay)	6.9

HIGH QC	
%Accuracy (between assay)	-3.6
%Precision (within assay)	6.8
%Precision (between assay)	6.7

Both accuracy and precision were within the required criteria as long as the calibrants linearity thus the QCs passed and the method was validated for serum AD.

5.3.6 Method validation for the quantification of serum TG

5.3.6.1 Calibration range

The lowest calibrant, at the LLOQ, had a percentage accuracy (within assay) <±20%, but the 2nd calibrant was above the acceptance criteria of <±15 %. The linearity of the calibration plots (r^2 value), for the 3 day validation of TG, from 0.05 to 2 ng/mL was 0.99, 0.99 and 0.98. The calibration range was thus accepted and the QC validation followed.

5.3.6.2 Quality controls validation

Table 5.15 Validation of serum TG for LOW, MEDIUM and HIGH QCs

	Day 1	Day 2	Day 3
LOW QC (ng/mL)	0.13	0.11	0.11
	0.13	0.10	0.12
	0.14	0.11	0.13
Mean (ng/mL)	0.14	0.11	0.12
% Accuracy	13.2	-12.4	-2.7
SD (ng/mL)	0.0	0.0	0.0
% CV	5.0	5.3	9.1

	Day 1	Day 2	Day 3
MEDIUM QC (ng/mL)	0.69	0.71	0.64
	0.69	0.69	0.67
	0.69	0.68	0.66
Mean (ng/mL)	0.69	0.69	0.65
% Accuracy	15.3	15.2	9.1
SD (ng/mL)	0.0	0.0	0.0
% CV	0.3	2.0	2.3

	Day 1	Day 2	Day 3
HIGH QC (ng/mL)	1.25 1.17 1.11	1.15 1.11 1.15	1.13 1.07 1.08
Mean (ng/mL)	1.18	1.14	1.09
% Accuracy	-5.9	-8.9	-12.7
SD (ng/mL)	0.1	0.0	0.0
% CV	5.7	1.9	3.1

Table 5.16 Validation parameters of serum TG for LOW, MEDIUM and HIGH QC

LOW QC	
%Accuracy (between assay)	-0.6
%Precision (within assay)	6.4
%Precision (between assay)	6.4

MEDIUM QC	
%Accuracy (between assay)	13.2
%Precision (within assay)	1.5
%Precision (between assay)	1.5

HIGH QC	
%Accuracy (between assay)	-9.2
%Precision (within assay)	3.6
%Precision (between assay)	3.6

Accuracy and precision were also within the acceptance criteria therefore the QCs passed and the method was validated for serum TG.

5.3.7 Method validation for the quantification of serum EG

5.3.7.1 Calibration range

The lowest calibrant, at the LLOQ, had a percentage accuracy (within assay) <±20%, but the other calibrants failed to pass the acceptance criteria of <±15 %. The linearity of the calibration plots (r^2 value), for the 3 day validation of EG, from 0. 5 to 2.5 ng/mL was 0.98, 0.96 and 0.92.

5.3.7.2 Quality controls validation

Table 5.17 Validation of serum EG for LOW, MEDIUM and HIGH QCs

	Day 1	Day 2	Day 3
LOW QC (ng/mL)	0.51	0.58	0.57
	0.46	0.58	0.59
	0.50	0.58	0.56
Mean (ng/mL)	0.49	0.58	0.57
% Accuracy	-18.1	-3.6	-4.3
SD (ng/mL)	0.0	0.0	0.0
% CV	6.1	0.8	3.2

	Day 1	Day 2	Day 3
MEDIUM QC (ng/mL)	1.32	1.11	1.00
	1.38	1.06	1.05
	0.99	1.17	1.05
Mean (ng/mL)	1.23	1.11	1.04
% Accuracy	-1.5	-11.1	-17.1
SD (ng/mL)	0.2	0.1	0.0
% CV	16.9	4.8	2.7

	Day 1	Day 2	Day 3
HIGH QC (ng/mL)	2.13	1.73	1.73
	2.31	1.89	1.92
	1.98	2.02	1.77
Mean (ng/mL)	2.14	1.88	1.81
% Accuracy	7.0	-6.0	-9.6
SD (ng/mL)	0.2	0.1	0.1
% CV	7.8	7.6	5.6

Table 5.18 Validation parameters of serum EG for LOW, MEDIUM and HIGH QC

LOW QC	
%Accuracy (between assay)	-8.7
%Precision (within assay)	3.4
%Precision (between assay)	3.2

MEDIUM QC	
%Accuracy (between assay)	-9.9
%Precision (within assay)	8.1
%Precision (between assay)	8.6

HIGH QC	
%Accuracy (between assay)	-2.8
%Precision (within assay)	7.0
%Precision (between assay)	7.0

Accuracy and precision for the QCs was within the acceptance criteria, but since some calibrants were not within the required accuracy range nor did the range show linearity (r^2 > 0.99), the method was not validated for serum EG.

5.3.8 Method validation for the quantification of serum TS

5.3.8.1 Calibration range

The lowest calibrant, at the LLOQ, did not have a percentage accuracy (within assay) <±20%, not did the 2nd calibrant of <±15 % (it was approximately ±20 %). The linearity of the calibration plots (r^2 value), for the 3 day validation of TS, from 0. 25 to 7.5 ng/mL was 0.99.

5.3.8.2 Quality controls validation

Table 5.19 Validation of serum TS for LOW, MEDIUM and HIGH QCs

	Day 1	Day 2	Day 3
LOW QC (ng/mL)	0.25	0.27	0.22
	0.24	0.27	0.25
	0.22	0.28	0.21
Mean (ng/mL)	0.24	0.27	0.23
% Accuracy	-5.8	9.5	-8.8
SD (ng/mL)	0.0	0.0	0.0
% CV	6.7	2.6	9.9

	Day 1	Day 2	Day 3
MEDIUM QC (ng/mL)	0.94	0.98	0.96
	0.96	1.02	1.03
	0.92	1.04	1.00
Mean (ng/mL)	0.94	1.01	1.00
% Accuracy	-6.1	1.3	-0.2
SD (ng/mL)	0.0	0.0	0.0
% CV	1.9	3.0	3.6

	Day 1	Day 2	Day 3
HIGH QC (ng/mL)	6.44	6.13	6.89
	6.26	5.89	6.60
	6.08	6.14	6.44
Mean (ng/mL)	6.26	6.05	6.65
% Accuracy	-3.7	-6.9	2.2
SD (ng/mL)	0.2	0.1	0.2
% CV	2.9	2.4	3.4

Table 5.20 Validation parameters of serum TS for LOW, MEDIUM and HIGH QC

LOW QC	
%Accuracy (between assay)	-1.7
%Precision (within assay)	6.4
%Precision (between assay)	6.2

MEDIUM QC	
%Accuracy (between assay)	-1.7
%Precision (within assay)	2.8
%Precision (between assay)	2.9

HIGH QC	
%Accuracy (between assay)	-2.8
%Precision (within assay)	2.9
%Precision (between assay)	2.9

Despite the fact that the lowest calibrants were not within the acceptance criteria, the range showed linearity and the QCs were all within the required parameters, therefore TS was considered to have been validated.

5.3.9 Method validation for the quantification of serum ES

5.3.9.1 Calibration range

The lowest calibrant, at the LLOQ, in the first day of validation did not have a percentage accuracy (within assay) <±20%, not did the 2nd calibrant of <±15 %. The linearity of the calibration plots (r^2 >0.99), for the 3 day validation of ES, from 0. 5 to 2.5 ng/mL was 0.93, 0.99 and 0.99.

5.3.9.2 Quality controls validation

Table 5.21 Validation of serum ES for LOW, MEDIUM and HIGH QCs

	Day 1	Day 2	Day 3
LOW QC (ng/mL)	0.46	0.63	0.60
	0.51	0.62	0.64
	0.46	0.58	0.56
Mean (ng/mL)	0.47	0.61	0.60
% Accuracy	-20.9	1.6	-0.2
SD (ng/mL)	0.0	0.0	0.0
% CV	6.1	4.0	6.4

	Day 1	Day 2	Day 3
MEDIUM QC (ng/mL)	0.95	1.03	0.99
	0.88	1.07	1.01
	0.80	1.03	1.01
Mean (ng/mL)	0.87	1.04	1.00
% Accuracy	-12.5	4.3	0.2
SD (ng/mL)	0.1	0.0	0.0
% CV	8.5	2.3	1.5

	Day 1	Day 2	Day 3
HIGH QC (ng/mL)	1.84	1.94	1.85
	1.72	1.88	1.74
	1.64	1.90	1.86
Mean (ng/mL)	1.73	1.91	1.81
% Accuracy	-13.5	-4.6	-9.3
SD (ng/mL)	0.1	0.0	0.1
% CV	5.9	1.4	3.8

Table 5.22 Validation parameters of serum ES for LOW, MEDIUM and HIGH QC

LOW QC	
%Accuracy (between assay)	-6.5
%Precision (within assay)	5.5
%Precision (between assay)	5.4

MEDIUM QC	
%Accuracy (between assay)	-2.6
%Precision (within assay)	4.1
%Precision (between assay)	3.9

HIGH QC	
%Accuracy (between assay)	-9.1
%Precision (within assay)	3.7
%Precision (between assay)	3.6

Despite the fact that the lowest calibrants were not within the acceptance criteria, the range showed linearity, apart from day 1, and the QCs were all within the required parameters, therefore ES was considered to have been validated.

5.3.10 Comments on the validation

It is not uncommon to have higher errors at the low end of the calibration range which, in principle, should not exceed an accuracy >20 %. In these situations, provided that there is linearity for the calibration range, the validation was considered to have passed.

For the analytes where these situations occurred or even in the case of ES where in day 1 the range did not show linearity, the analyte was monitored carefully in each run to verify if linearity or differences in accuracy in the mid and high range occurred.

Samples T9M-S-8U-2h and T9M-S-8U-6h (Appendix 8.32) were outliers in that they did not have a quantifiable TG concentration and the TS concentrations were higher than the group mean (approximately 27 times more).

5.3.11 Serum sample analysis

Statistical treatment was performed as for the previous chapters. The post-administration data was averaged irrespective of gender, time or units and the data was normalized using proportion increase against pre-administration as follows:

Equation 5.6 Normalization of values for statistical treatment

A similar situation as to what happened in previous chapters occurred with this analysis, which was that the samples collected at 24 h and 48 h had already reached baseline values. A comparison between baseline value (baseline sample taken before alcohol ingestion) and these two samples was performed to ascertain if the response was different to the 4 and 8 units administered. The results were not statistically significant with serum T having a p-value =0.239 and the value for AD being p-value =0.577 and hence the last two measurements (24 and 48 h) were excluded from the statistical treatment. As an added test of reliability of the baseline values, the mean of the values determined in the pre-administration, 24 h and 48 h samples was used as the baseline value.

Except with T, all the analytes had certain samples in which the concentration obtained was below the LLOQ (Table 5.23).

Table 5.23 Percentage of eugonadal samples with concentrations below the LLOQ, in LC-MS/MS

Steroid	Percentage of samples < LLOQ			
	Males 4 units	Females 4 units	Males 8 units	Females 8 units
T	-	-	-	-
E	36 %	100 %	52 %	98 %
AD	9.7 %	-	17 %	0.05 %
TG	43 %	35 %	47 %	37 %
TS	35 %	92 %	27 %	91 %
ES	100 %	100 %	96 %	100 %

In a series of samples from a particular volunteer, if there were more than 25 % of the post-administration samples below the LLOQ, it was decided not to perform any statistical analysis since increases would most likely not be verified.

The difference in quantifiable TG between volunteers may be related to an absence of the UGT2B17 enzyme (caused by a double deletion in the UGT2B17 gene) responsible for the conjugation of T. Genetic tests on these samples have not been performed yet due to time constraints.

Statistical analysis was done with T and AD in serum samples. Since we were interested in concentration differences and not absolute values *per se*, where samples had values < LLOQ,

after discussion with the statistician, we were advised to change that value to half of the LLOQ of the analyte for statistical treatment purposes.

The serum concentrations for males and females after 4 units may be found Appendix 8.30 and Appendix 8.31, respectively. The concentrations for males and females after 8 units follow in Appendix 8.32 and Appendix 8.33.

5.3.12 Serum T concentration analysis

The normalized mean serum T response (proportion increased when compared with pre-administration) was significantly different for 4 and 8 units ($p < 0.05$) across time. There were gender differences ($p < 0.05$) and although females showed a higher response averaged over time, that response was not significantly different between 4 and 8 units ($p > 0.05$). At 4 units, serum T increases up to 3 h then it decreases reaching baseline at 5 h. After 8 units, it increases up to 6 h having reached baseline at 24 h.

Since there were gender differences but no dose difference averaged over time and gender ($p > 0.5$) the data will be presented in a graph with the mean normalized values for each collection point, averaged for the two units but independent of gender (Figure 5.25).

The concentration of T in serum increased in the female volunteers following alcohol administration. By contrast, there was a decrease in the male volunteers.

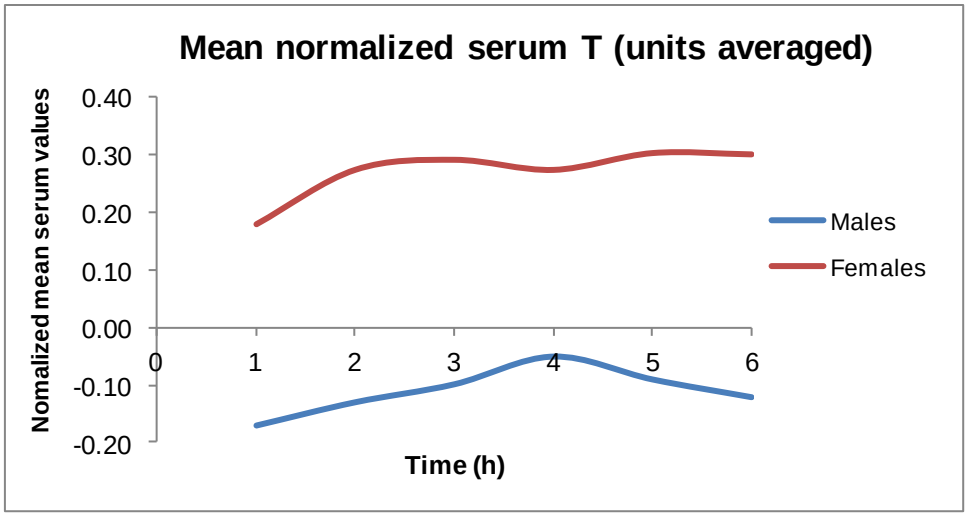


Figure 5.25 Normalized mean serum T values, for males and females, units averaged

The raw values (not normalized), after 8 units, for females and males have also been plotted and may be found in Figure 5.26. Since the serum T concentration in females is approximately one tenth that of males, these were plotted on a secondary axis on the right hand-side.

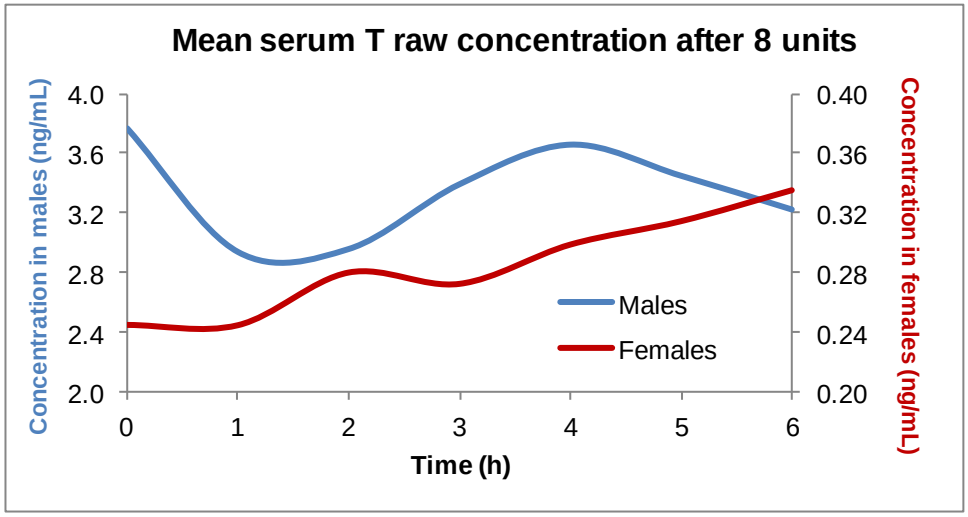


Figure 5.26 Mean serum T raw concentrations, for males and females, after 8 units

5.3.13 Serum AD concentration analysis

Statistical treatment of serum AD values showed that there were no gender differences ($p > 0.05$) after data normalization related to pre-administration values. The serum AD response was different for 4 and 8 units ($p < 0.05$) averaged over time and gender. The type of response that females and males show, averaged over time is different when the units increase ($p < 0.05$). There were changes with time ($p < 0.05$), and the AD concentration time plot for 4 and 8 units (gender averaged) was different ($p < 0.05$), showing a decrease in AD concentration.

Since the unit dose was significantly different but there were no gender differences, the data will be presented in a graph with the mean normalized values for each collection point, averaged over gender for the two different units (Figure 5.27).

There was a decrease after 4 and 8 units which was more accentuated in the latter. The concentration started increased after 1 h with 4 units and after 2 h with 8 units. However, that increase never reached baseline during the 6 h collection.

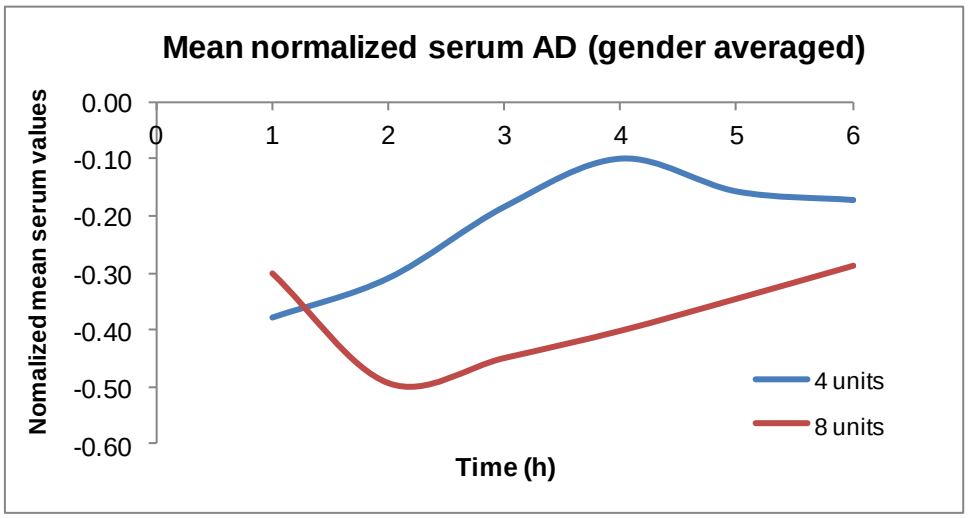


Figure 5.27 Normalized serum AD values, for 4 and 8 units, gender averaged

The mean raw concentrations for serum AD, after 8 units, for males and females may be found in Figure 5.28, where a decrease in concentration is observed.

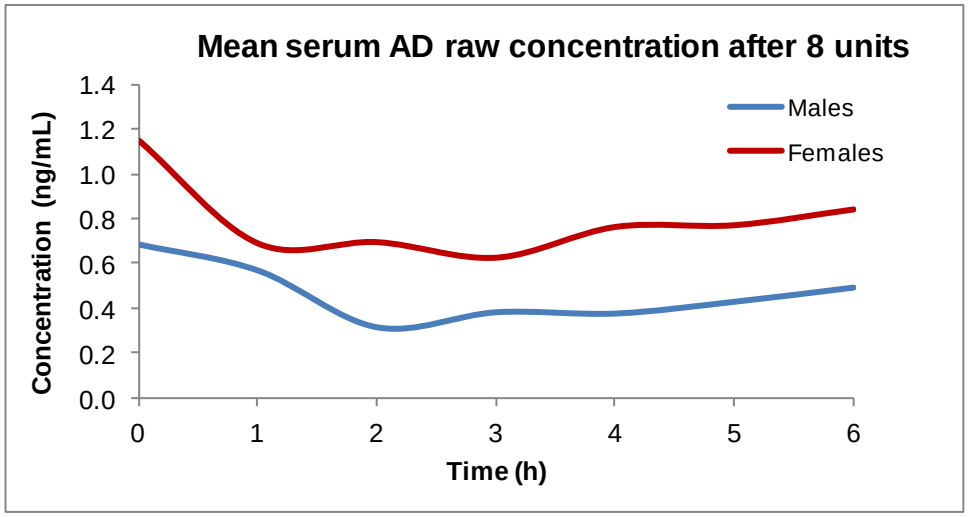


Figure 5.28 Mean serum AD raw concentrations, for males and females, after 8 units

5.3.14 Correlations

Correlation statistics were applied to investigate whether there was any relationship between serum T and AD, serum T and urinary T/E, serum T and the serum alcohol concentration, as well as serum AD and serum alcohol concentration.

Administration of alcohol to females resulted in an increase in serum T and a fall in serum AD. The correlation between serum T and AD, for females taking 4 units of alcohol, showed a significant difference between subjects ($p < 0.05$), but there was no effect overall possibly due to the fact that each individual is so different that masks the overall effect. The suppression of AD has an effect in the interaction between AD and serum T but it is a subject-based one.

With administration of 8 units of alcohol to females, overall there is a correlation that is significant, but it varies between subject, not being consistently negative meaning that it is not always the case that serum T increases when serum AD decreases.

For males, with serum T and serum AD decreasing, after 4 units and 8 units of alcohol, there is barely any correlation.

Serum T correlated with urinary T/E did not show any significant effect for females and males, after 4 units. After 8 units, there was an overall correlation but even the best-fitted trendline was showing values that were spread out from it.

Considering serum T and serum alcohol concentration, there was an overall positive correlation but each individual showed different behaviours, some showing a negative correlation. This may be because the 'clock-time' for the metabolism of these analytes is different and we are analysing them at the same time.

Finally, considering serum AD and serum alcohol concentration there was an overall negative correlation, which was clearer than with serum T.

Most likely, T and AD are being necessary in other parts of metabolism that are not exclusive to alcohol, and the rate at which this is occurring is individual to each analyte. There is also the possibility of it being a 'lag' between the time alcohol enters the body and the time that it starts having an effect in the desired analytes. The metabolic rates of alcohol elimination have a considerable inter-individual variability (Eckardt et al., 1998). This is most likely due to allelic variants of the genes that encode ADH and ALDH. Functional polymorphisms in these genes have shown to increase the variance amongst individuals and this variance may be related to this 'lag'.

The same happening when correlating serum T with T/E, with the analytes being produced and excreted at different rates.

It is not possible, in most cases, to have clear information since it would be necessary to have more data points to better correlate our data.

In summary, it is not straightforward to correlate this data as more data points would have been necessary. Our initial experiment design was not set-up to determine these measurements but to verify if there were significant changes in the desired analytes due to alcohol, which was achieved.

5.4 Conclusions

The analysis of serum samples via LC-MS/MS after 4 and 8 units of alcohol, (approximately 0.5 and 1.0 g/kg) in eugonadal males and females, shows that males and females are affected differently. The serum T concentration time pattern varies with the different units, and between females and males.

This agrees with the findings by Sarkola *et al.* where after 0.5 g/kg in females, there was an increase in serum T which was significantly different (Sarkola et al., 2001). It also agrees with the findings of Frias *et al.* in which alcohol suppressed serum T in males (Frias et al., 2002)

However, the increases observed in eugonadal females in this study were more modest in comparison to the study by Sarkola *et al.* where a blood alcohol concentration of 5 mmol/L reflected an increase in plasma T from 0.14 ng/mL to 0.6 ng/mL (Sarkola et al., 2001) when compared to an increase from 0.26 to 0.34 ng/mL with a serum alcohol concentration of 3.2 ng/mL. Although the analysis of alcohol concentration was undertaken with different matrices, it has been shown that serum and plasma are composed of approximately 92 % w/w water when compared to 80 % w/w in whole blood. If assumed that there are no differences in matrix (serum and plasma), the ratio of plasma/whole blood has been justified as 1.2 (Moffat et al., 2011, Iffland et al., 1999).

The differences observed in serum T may be related to the analytical technique employed. In the study by Sarkola *et al.* the T concentrations were measured via immunoassay which may have cross-reacted with TG, thus increasing the serum T concentration. With mass-spectrometry, the technique used in this research study, such does not happen.

The statistically significant change in AD concentrations shows that there is evidence to relate its acute decrease to alcohol.

The hypothesis that ethanol administration decreases serum AD by enzyme suppression thus increasing serum T was supported. The next, and final, chapter will verify whether alcohol has an effect on clearance of T (either hepatic metabolism or renal clearance) by analysis of data in hypogonadal men.

Chapter 6 INVESTIGATING THE EFFECT OF ALCOHOL ADMINISTRATION ON METABOLISM AND EXCRETION OF T (IN HYPOGONADAL MEN RECEIVING HORMONE REPLACEMENT THERAPY)

6.1 Introduction

This final data chapter aims to verify whether the ingestion of alcohol has an effect on the clearance of T (either by hepatic metabolism or renal clearance) by analysis of data in hypogonadal men.

Investigating the effects of ethanol administration using as a model hypogonadal men receiving testosterone replacement therapy, will help to clarify the effect of the liver (or kidneys) to any transient effects in serum testosterone, as there should be a negligible contribution to circulating T from the testis.

Clearance, as described in Chapter 1 is most commonly described as the volume of blood completely cleared of a substance (e.g. a drug) as measured in unit time (L/day), by a certain organ such as the liver or kidneys. Elimination is the excretion of the unchanged or metabolised drug via the urine, gut contents, or sweat.

6.1.1 Hypogonadism & hormone replacement therapy

Hypogonadism occurs when the sex glands (testes or ovaries) produce little or no hormones. In hypogonadal men, their testes produce little or no T.

Male hormone replacement therapy is available to help restore androgens to their reference concentrations thus bringing the body to its normal physiological status restoring the secondary sexual characteristics (beard growth, body hair), somatic development (deepening of voice, increase in fat-free mass, bone mineral density and sexual function) (Snyder et al., 2000, Griffin and Wilson, 1992).

There are two types of hypogonadism: hypergonadotropic and hypogonadotropic.

Hypergonadotropic hypogonadism is related to primary gonadal disorders. There is a deficiency of steroid gonadal production (low serum T), which, due to the negative feedback mechanism in the HPG axis, leads to high concentrations of circulating LH and FSH. This type of primary gonadal failure is most commonly related to genetic disorders, such as Klinefelter syndrome in men and Turner syndrome in women (Grumbach and Styne, 1992, Dobs et al., 2005).

In Klinefelter syndrome, there is an extra X chromosome and males are referred to as being 47 XXY. This syndrome occurs in 1 out of 1,000 men, and often results in small, firm testes, impaired spermatogenesis and a male phenotype of eunuchoid resemblance (Grumbach and Styne, 1992).

In Turner syndrome, or gonadal dysgenesis, women lack one of their X chromosomes and are referred to as being 45 XO. This disorder is found in 1 out of 2,500 births and is characterized by short stature, sexual infantilism, infertility and low production of sexual hormones.

In hypogonadotropic hypogonadism, or secondary hypogonadism, there is a pituitary deficiency which causes insufficient secretion of LHRH and GnRH, which affects the production of LH and FSH thus influencing the production of T and spermatogenesis. Gonadotropin deficiencies may be caused by CNS disorders (tumours, congenital malformations), chronic systemic disease and malnutrition, hypothyroidism, diabetes or Cushing's disease, just to name a few.

Formulations and routes of delivery for androgen replacement therapy for hypogonadal men vary and may be taken orally, as a gel, a patch, a pellet or an injection, the latter being the most common one, in the form of an ester. Esterification of testosterone (17β-hydroxyl group) renders it less polar and more lypophilic, and slowly absorbed by the body.

The gel and patch are transdermal preparations of T and recent studies have shown that T gel replacement improved sexual function and mood, lean mass and muscle strength, and decreased fat mass in hypogonadal men with less skin irritation than T patch (Wang et al., 2000). Although a T gel application could have been the therapy replacement of choice for the patients in this study, these transdermal formulations are designed to be applied daily and there was some concern that volunteers may forget to do so, i.e. a non-compliance issue.

Testosterone injections composed of only one T ester was the chosen type of hormone replacement for this study. After injection, serum T concentration rises up to almost its upper reference limit (~10 ng/mL) or even beyond to supraphysiological concentration, followed by a decrease to eugonadal levels (Dobs et al., 2005). The chosen injection was Nebido®, composed only of T undecanoate.

6.1.2 Hypogonadism & urinary T/E analysis after alcohol

In healthy men, alcohol appears to have little effect on serum testosterone and the increase in the urinary testosterone/epitestosterone (T/E) ratio is much smaller compared to women. The data are limited and it is difficult to elucidate whether the effect in men is due simply because of inhibition of the hepatic enzyme 17β-hydroxysteroid dehydrogenase (17 β-HSD2), as reported in women (Sarkola et al., 2001), and verified in our study, or whether there is an acute effect on testicular production of testosterone (T) as opposed to a chronic effect that appears to have an effect on the hypothalamic – pituitary – gonadal axis by inhibiting testicular production of T (Ellingboe and Varanelli, 1979). Using as a model hypogonadal men which are on hormone replacement therapy, thus excluding any negligible contribution to T production from the testis, will elucidate the effect of the liver (or kidneys) to any changes in serum testosterone.

For this part of the research study, a collaboration with the Medical Research Council (MRC) Simpson's Centre for Reproductive Health in the University of Edinburgh, with Dr. Richard Anderson, Dr. Jyothis George and Mr. Nick Malone was established.

Three volunteers were recruited, and the hormone replacement they were being administered was an intra-muscular injection of Nebido®. The volunteers were asked to attend the study in two different occasions. One when they would be in a steady state period regarding their serum T concentration (within the reference range); and the other when they would be at a serum concentration greater than the upper reference limit (> 10 ng/mL) which happens up to one week after Nebido® injection (Electronic Medicines Compendium, 2011, Dobs et al., 2005). The reason for investigating both are that the latter would mimic a population of healthy men (athletes) that would have recently been administered a supraphysiological dose of T.

The data analysis in this chapter is was performed using the urinary GC-MS analysis described in Chapter 3 and the serum LC-MS/MS analysis as described in Chapter 5.

6.2 Material and methods

6.2.1 Materials

For urinary analysis of T/E, T concentration, E concentration and 5α-DHT concentration please see Chapter 3.

For serum analysis of T and AD, please see Chapter 5.

6.2.2 Clinical study and sample collection

Ethical approval was sought and given, for the recruitment of 3 male hypogonadal patients to whom 8 units of alcohol were administered and blood and urine collected were performed post-alcohol ingestion.

The samples were labelled with an alphanumeric code, e.g. TαM-β8U-δχ, where α is the number of the volunteer, β would either be SS for steady state or SP for supra-physiological, δ the letter indicating the type of sample (U for urine and S for serum), and χ the time of collection.

The volunteers followed the same inclusion criterion as the eugonadal men in the main study, described in Chapters 3-5, for example the alcohol abstinence for 3 days before the study. However, the upper age limit was raised to 50 years, it was reasoned that there should be no difference between a younger liver and the liver of a man slightly older. An exclusion criterion was a body mass index (BMI) <30 kg/m^2. The volunteers were asked to provide blood and urine at the same time intervals as in the eugonadal study (Chapters 3, 5), except 48 h and 72 h, which we have decided to exclude for this study assuming the values of the metabolites of interest had already reached baseline similar to what was verified in the eugonadal study.

Serum was analysed by liquid-chromatography tandem mass-spectrometry (LC-MS/MS) to measure T, E, its glucuronides and sulphates and androstenedione. The urine samples were analysed by GC-MS to measure the T/E ratio, concentration of T, E and 5α-DHT.

Due to time constraints, the serum alcohol measurements were not performed in time for inclusion in this chapter.

The statistical treatment was performed using the software Statistical Package for Social Sciences (SPSS®), version 18, from SPSS® Inc, Chicago, IL, USA. The results were statistically treated by using a general linear model with repeated measures to test for any effects of alcohol in urinary T/E and the analytes of interest in urine and serum. The significance limit was set to p-value <0.05.

6.3 Results

6.3.1 Sample analysis and challenges encountered

Statistical treatment was performed as for the previous chapters. The post-administration data was averaged irrespective of the group (steady state or supraphysiological) and time and the data was normalized using proportion increase against pre-administration as follows:

Equation 6.1 Normalization of values for statistical treatment

The samples collected at 24 h had already reached baseline values (Appendix 8.34 and Appendix 8.35) and a comparison between baseline value (baseline sample taken before alcohol ingestion) and this sample was done to ascertain if the response was different. The results were not statistically significant with p-value >0.05. Hence, the last measurement was excluded from the statistical treatment. As an added test of reliability for our baseline values, the mean of pre-administration and 24 h sample was used as the baseline value.

Urinary T/E, and the concentrations of the previously validated steroids (T, E and 5α-DHT) were calculated. Urinary concentrations of analytes vary between individual and are dependent of urinary density and specific gravity (SG). Only values normalized for a SG of 1.020 can be compared and therefore the SG of all urine samples was measured and the concentration of steroid adjusted according to the following equation:

Equation 6.2 Adjusted concentrations

In two urine samples analysed for E concentration samples were below the LLOQ and therefore they were changed to half the value of the LLOQ's concentration, 0.5 ng/mL for statistical purposes (Appendix 8.34 and Appendix 8.35).

One volunteer in the supraphysiological phase had urinary T concentration values above the highest calibrant (160 ng/mL) without overloading the column (verified by chromatogram analysis). Since the purpose of this study is to verify if there is an increase in concentration, rather than measuring its exact value, due to time constraints it was not possible to dilute the sample in half, by extracting 1 mL rather than 2 mL, and because the calibration curve was linear (r^2 >0.99) the values higher than 160 ng/mL were included in our data treatment.

Considering serum, the measured concentrations are presented in Appendix 8.36 (steady state volunteers) and Appendix 8.37 (supraphysiological phase) the mean value of serum T concentrations in pre-administration samples in the volunteers in the steady state was 5.7 ±2.6 ng/mL and in the supraphysiological phase it was 8.3 ±0.8 ng/mL. Although the supraphysiological phase is not above the serum T's concentration upper reference limit of 10 ng/mL when compared to the steady state pre-administration mean concentration, there was a 1.5-fold increase and thus these volunteers were considered as supraphysiological. Although the volunteers undertook the second administration of alcohol within a week of having had their Nebido® injection, the reason for the serum T not to be above the upper reference limit may be that the T undecanoate present in the injection stabilizes faster (Electronic Medicines Compendium, 2011) and most likely not within a week as mentioned in the paper by Von Eckardstein *et al*. (Von Eckardstein and Nieschlag, 2002)

The concentration of TG was above the LLOQ, unlike in samples collected from the eugonadal volunteers (Chapter 5). This seemed reasonable since the mean pre-administration concentrations of T in eugonadal men (8 units) were 3.31 ± 1.22 ng/mL, so 1.7-fold less than hypogonadal men in their steady state. Statistical analysis was not performed in this analyte since it was not thought to be relevant for this hypothesis.

The normalized data is presented as SPSS graphs, except for the serum TG data.

6.3.2 Urine analysis

6.3.2.1 Urinary T/E

Urinary T/E for steady state and supraphysiological phase, normalized data (proportion increase from baseline) was plotted in Figure 6.1.

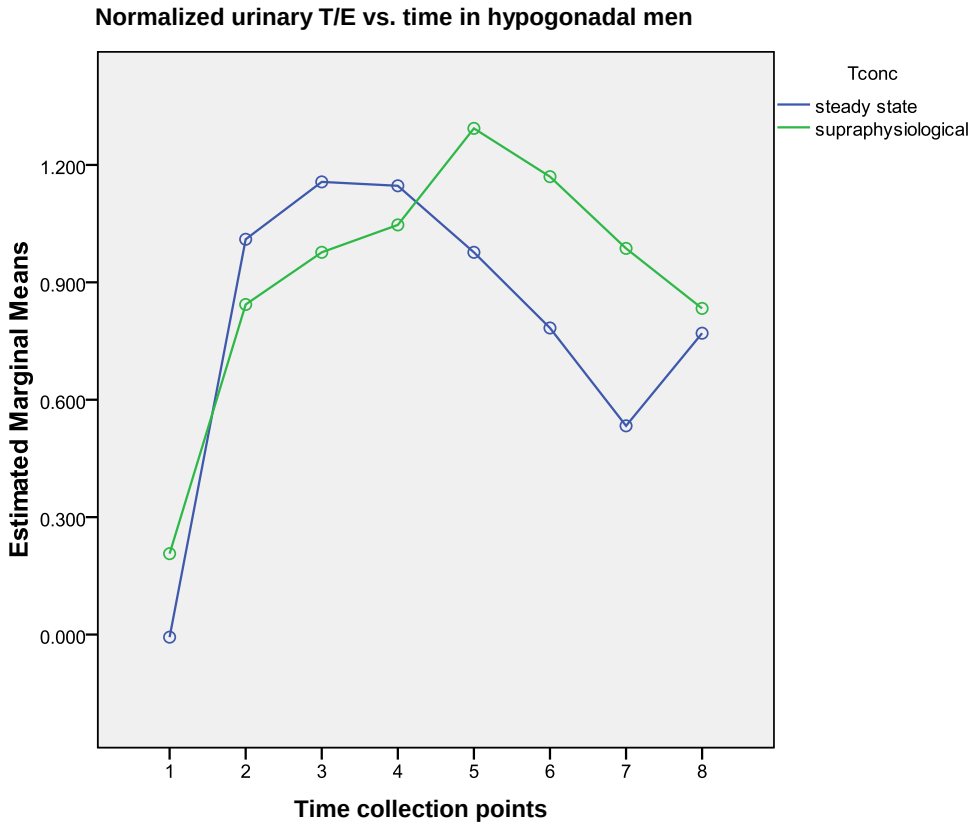


Figure 6.1 Normalized urinary T/E in hypogonadal men after 8 units of alcohol

The raw values may be found in Figure 6.2. The WADA threshold of 4 is also plotted for easier analysis.

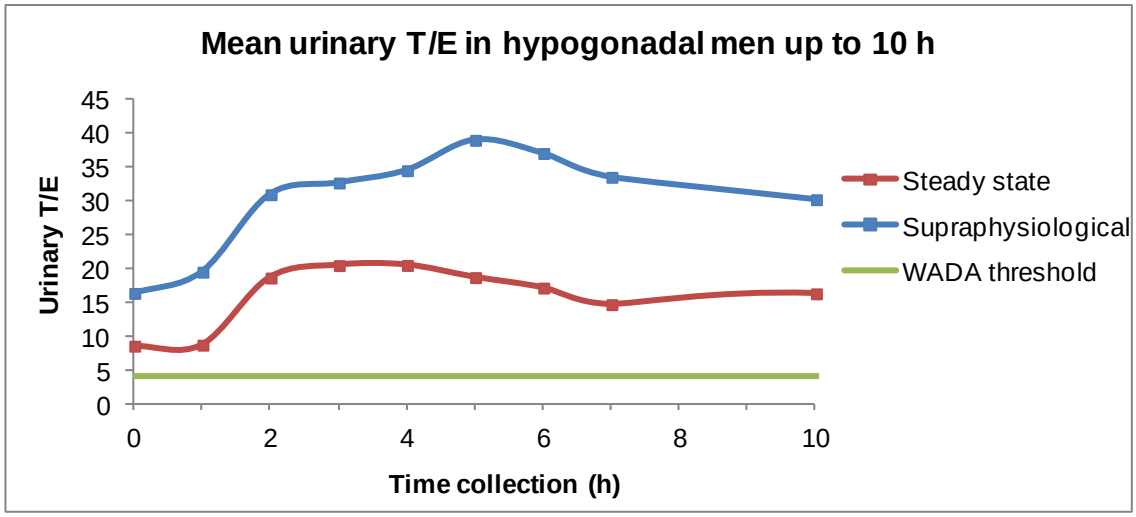


Figure 6.2 Mean urinary T/E in hypogonadal men in steady state and supraphysiological phase up to 10 h, after 8 units of alcohol

All the samples showed a pre-administration T/E above the WADA threshold, and an increase is verified up to 5 h in the supraphysiological phase, and up to 4 h in the steady state. After this, the urinary T/E starts decreasing until it reaches baseline values at 24 h.

Statistical treatment shows that there were changes over time due to alcohol intake ($p < 0.05$), averaged over the two groups, and that there were no statistically significant differences between the groups ($p > 0.05$) and T/E over time behaved similarly for the two groups ($p > 0.05$).

In Appendix 8.34 and 8.35, where the urinary T/E, the urinary T, E and 5α-DHT concentrations may be found, there are cases where the E concentration is 0.5 ng/mL. That was due to the sample being too diluted and hence below the LLOQ. For statistical purposes, the value given to such samples was half of the LLOQ because we are interested in changes in concentration rather than the concentration *per se*.

6.3.2.2 Urinary T concentration

Urinary T normalized concentration was plotted in Figure 6.3.

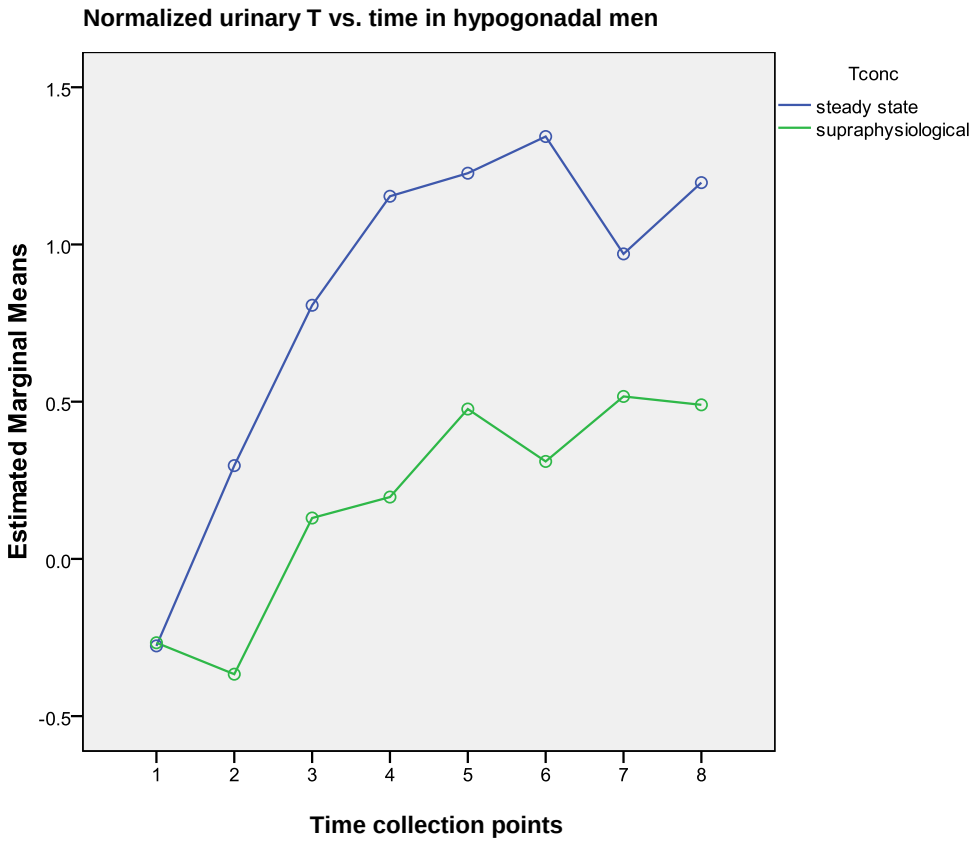


Figure 6.3 Normalized urinary T values in hypogonadal men after 8 units of alcohol

The raw concentration (ng/mL) has been plotted in the figure below (Figure 6.4). The WADA threshold for T concentration has also been plotted and as one can see, in the supraphysiological phase after 6 h and until just before 10 h, the concentration goes above the threshold.

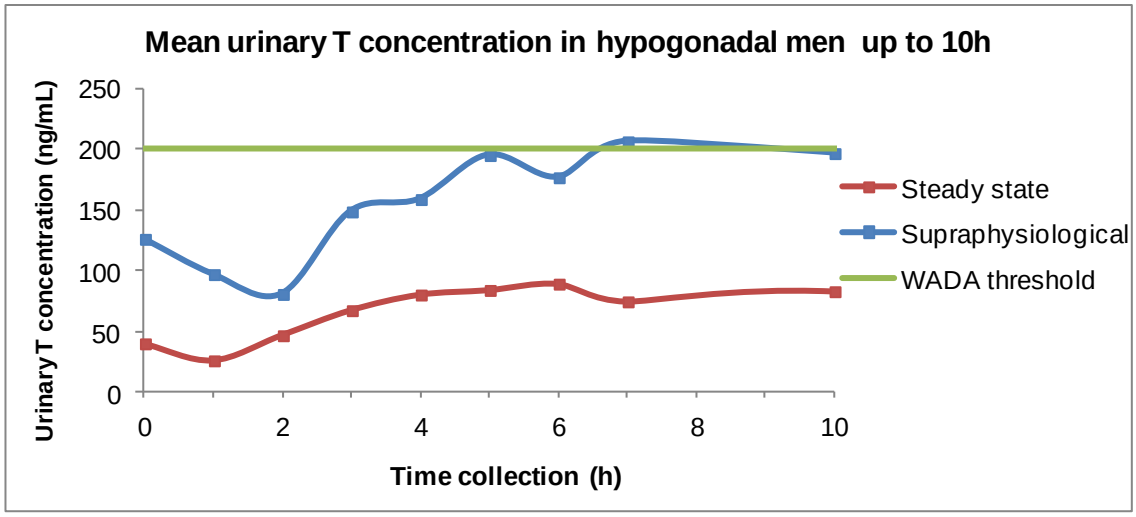


Figure 6.4 Mean urinary T concentration (ng/mL) in hypogonadal men in steady state and supraphysiological phase up to 10 h, after 8 units of alcohol

After statistical treatment, the increase in concentration of T over time, averaged over the groups, was statistically significant ($p < 0.05$). The T concentration in both groups behaves differently ($p < 0.05$), and the time pattern was similar for both groups ($p > 0.05$), as one can see there is an increase up to 10 h in both groups, after which it decreases to baseline values.

6.3.2.3 Urinary E concentration

The concentration of urinary E (ng/mL) was measured and the normalized data are presented in Figure 6.5.

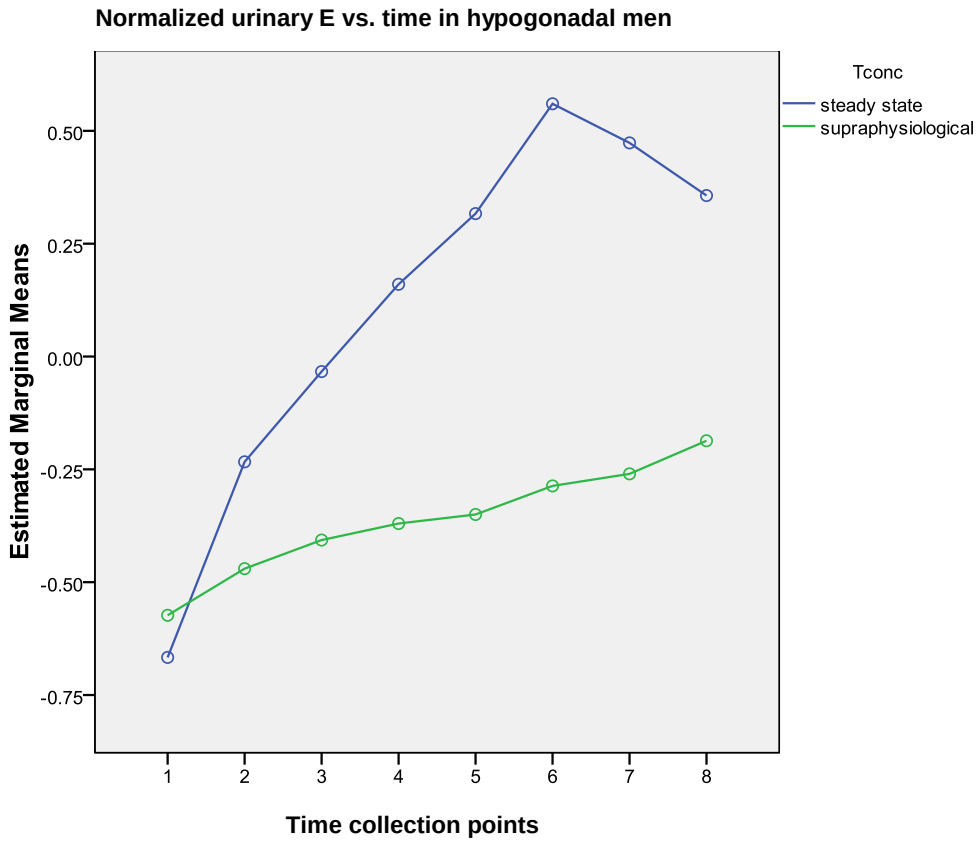


Figure 6.5 Normalized E values in hypogonadal men after 8 units of alcohol

The WADA threshold is the same as for T, which is 200 ng/mL. However, this was not plotted in the graph since the difference between the highest measured E concentration and the threshold concentration was very different and it was not thought to be necessary. The raw E concentration may be found in Figure 6.6.

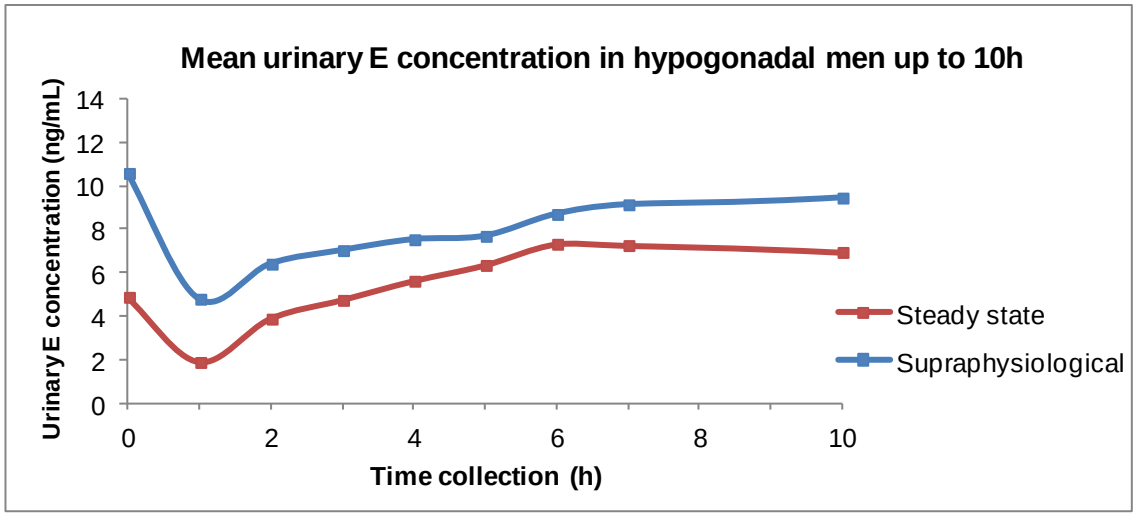


Figure 6.6 Mean urinary E concentration (ng/mL) in hypogonadal men in steady state and supraphysiological phase up to 10 h, after 8 units of alcohol

From the analysis of the graph, one can realise that there is a decrease after alcohol intake but after 1 h, the E concentration starts increasing until it reaches baseline values. That differences in E concentration over time are statistically significant (p-value <0.05), averaged over both groups. The E concentration difference is similar in both groups (p -value >0.05), and the time pattern showed that both groups behaved differently (p-value <0.05), with E concentrations from the steady state going above the baseline, which does not happen in supraphysiological phase. From looking at the figure, it seems as if E is being formed from T since E is at a bigger concentration in the supraphysiological phase than in steady state. However, such is not likely since the main pathway for the biosynthesis of E is from conversion of DHEA to AD and then reduction to E as discussed in Chapter 1, section 1.3.3 (Biosynthesis of Epitestosterone) (Bellemare et al., 2005).

6.3.2.4 Urinary 5α-DHT concentration

In what concerns the normalized values of urinary 5α-DHT, these were plotted in

Figure 6.7.

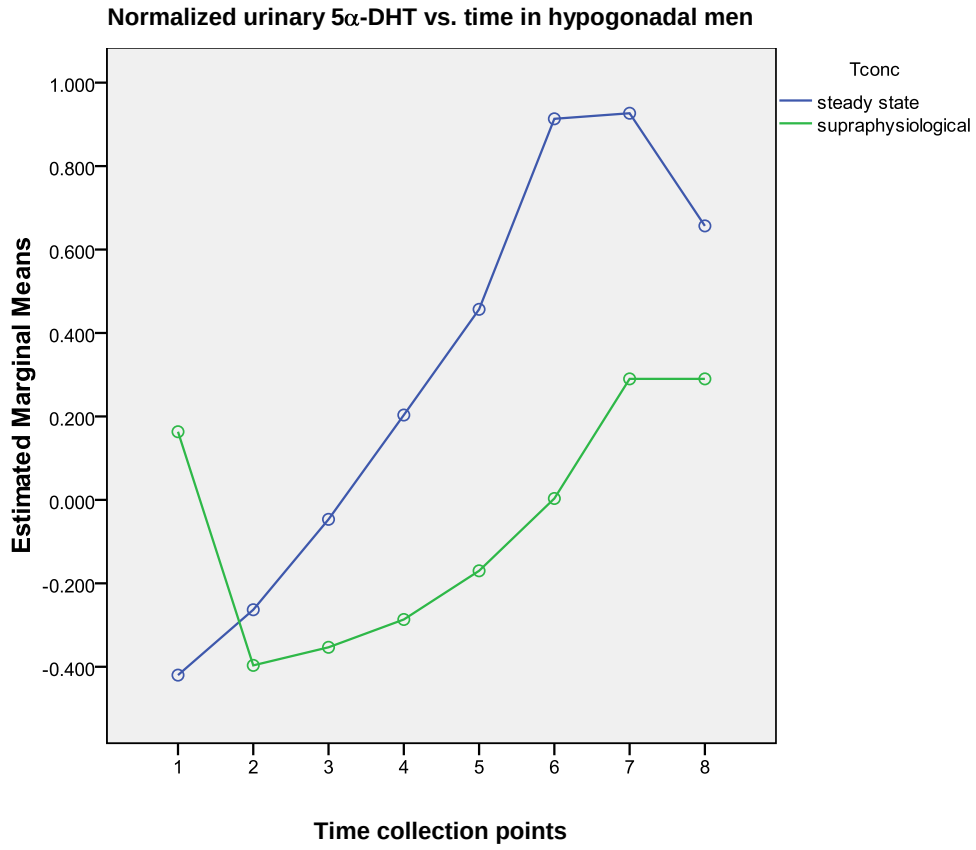


Figure 6.7 Normalized urinary 5α-DHT values for hypogonadal men after 8 units of alcohol

Regarding the urinary concentration of 5α-DHT (ng/mL), the respective graph may be found in **Error! Reference source not found.**.

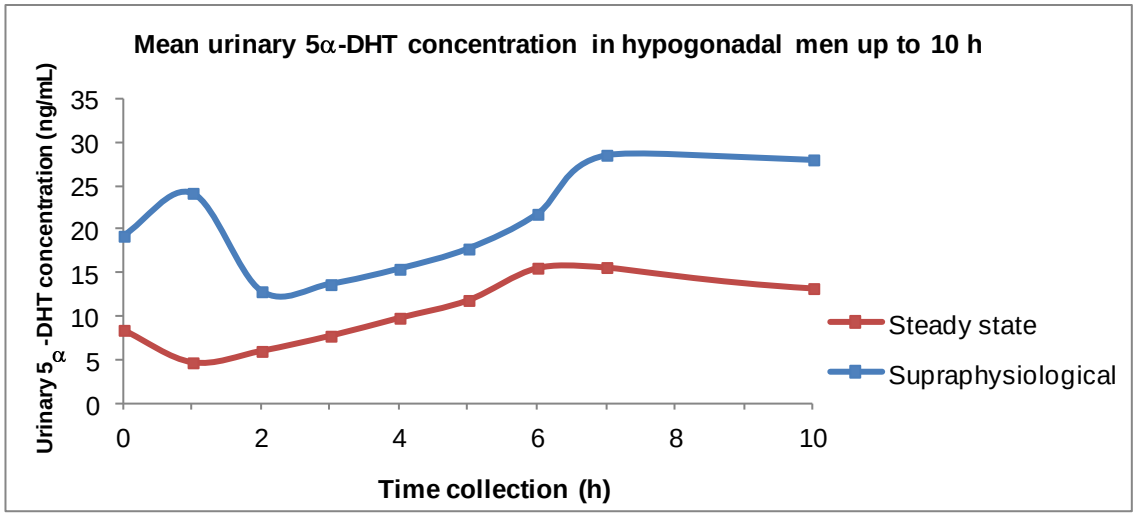


Figure 6.8 Mean urinary 5α-DHT concentration (ng/mL) in hypogonadal men in steady state and supraphysiological phase up to 10 h, after 8 units of alcohol

The difference in concentration over time after 1 h was statistically significant (p-value <0.05), averaged over both groups, and there was a difference in 5α-DHT response in the two groups (p-value <0.05). The time pattern was different for both groups (p-value <0.05), with the steady state concentrations increasing up to 6 h after 1 h, then decreasing up to 10 h but still remaining above baseline. With supraphysiological concentrations, there is an increase after 1 h, then a decrease down to 2 h that goes below the baseline, after which it starts increasing up to 7 h, going above the baseline at this time point. After 7 h, the concentration decreases but it is still above the baseline values.

The adjusted concentrations may be found in Appendix 8.3. The concentration for T17M-U-1h may be considered as an outlier.

6.3.3 Serum sample analysis

6.3.3.1 Serum T concentration

The serum T normalized values, where it is possible to see the proportion difference from the baseline (pre-administration) concentration was plotted in Figure 6.9.

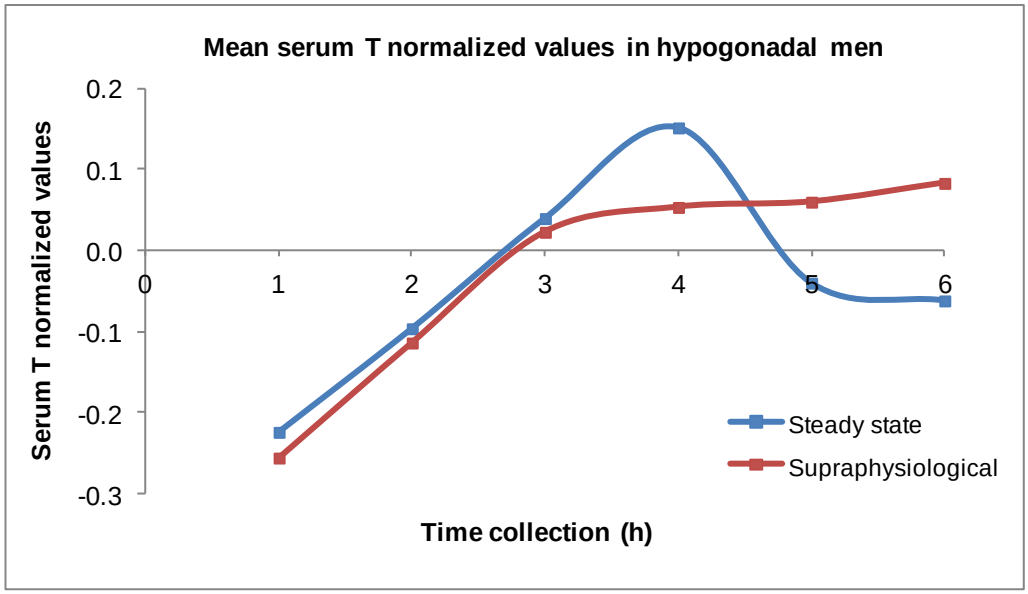


Figure 6.9 Mean serum T normalized values in hypogonadal men, after 8 units, up to 6h

The raw values were also plotted Figure 6.10.

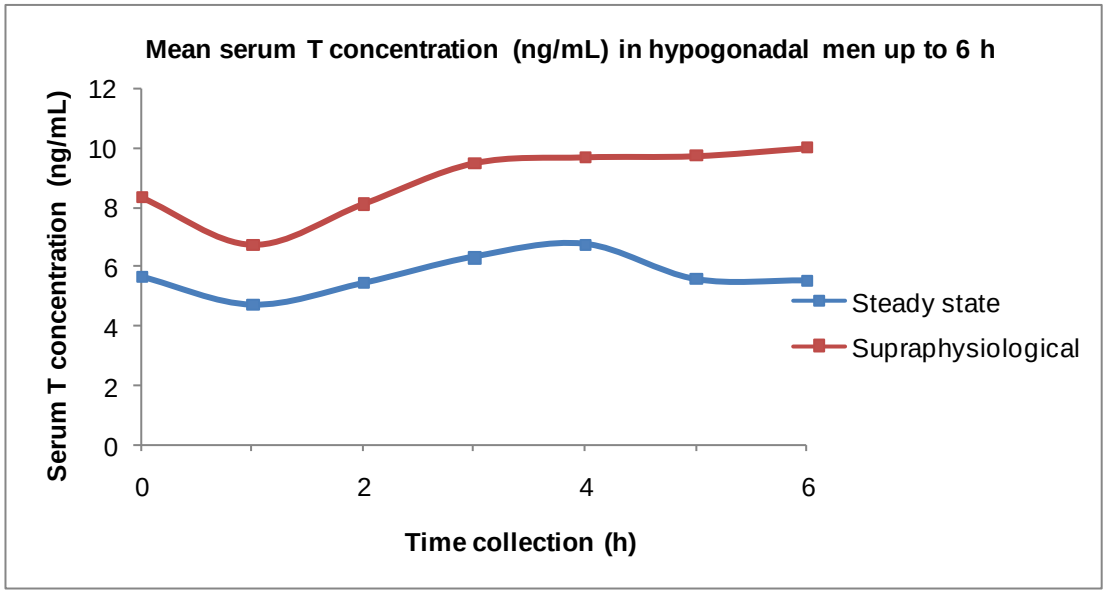


Figure 6.10 Serum T concentrations (ng/mL) in hypogonadal men, up to 6 h, after 8 units of alcohol

The decrease in serum T is statistically significant, over time, averaged over both groups (p-value <0.05). Both groups behave similarly (p-value >0.05), and the time pattern for both groups also showed a similar behaviour.

6.3.3.2 Serum AD concentration

The serum AD concentration was plotted in Figure 6.11.

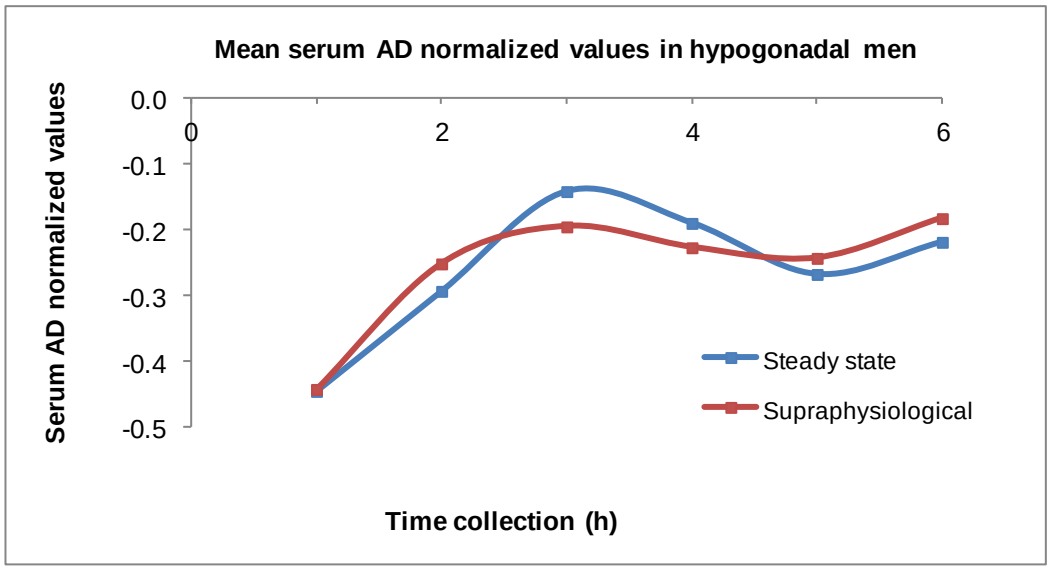


Figure 6.11 Serum AD normalized values in hypogonadal men after 8 units, up to 6 h

The raw concentrations may be found in Figure 6.12.

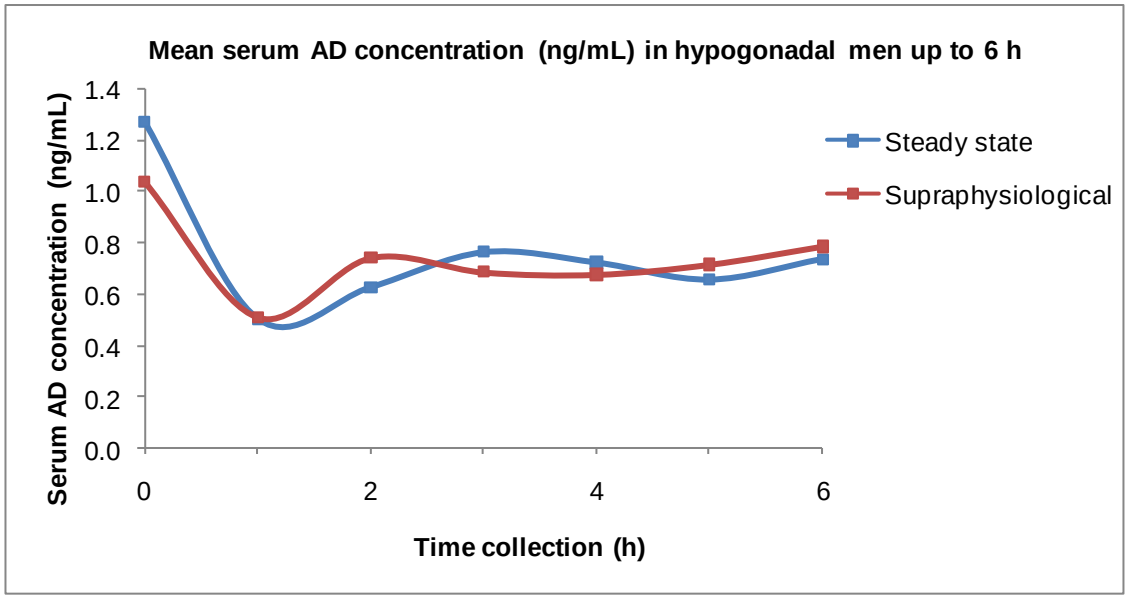


Figure 6.12 Mean serum AD concentration (ng/mL) in hypogonadal men, up to 6 h, after 8 units of alcohol

There is a decrease in serum AD which was not statistically significant (p-value >0.05) over time, averaged over both groups. There was no difference between both groups (p-value >0.05) and the time pattern showed a similar behaviour (p-value>0.05) with both groups decreasing up to 1 h, then increasing up to 2 h in the supraphysiological phase and to 3 h in the steady state, then decreasing up to 4-5 h, and increasing after that without ever reaching baseline values.

6.3.3.3 Serum TG concentration

The concentration of serum TG was above the LLOQ in all the samples, unlike in samples collected from the eugonadal volunteers (Chapter 5) where approximately 50 % were below the LLOQ and hence no statistical treatment was performed on those samples.

Testosterone glucuronide is one of testosterone's phase 2 metabolites, the other being testosterone sulfate. The mean pre-administration concentration of serum T, in eugonadal men was 3.9 ± 1.2 ng/mL, so 32 % less than hypogonadal men in their steady state (5.7 ±2.6 ng/mL) and 53 % less than supraphysiological hypogonadal men (8.3 ±0.8 ng/mL). Due to the higher T concentration in hypogonadal men when compared to eugonadal, it seemed reasonable that

serum TG was presented in these volunteers at a higher concentration, which was possible to quantify.

The serum TG concentration was plotted in Figure 6.13 as normalized values, showing a significant increase following alcohol administration over time (p-value <0.05), averaged for both steady phase and supraphysiological phase. There was no difference in TG response between both groups (p-value >0.05).

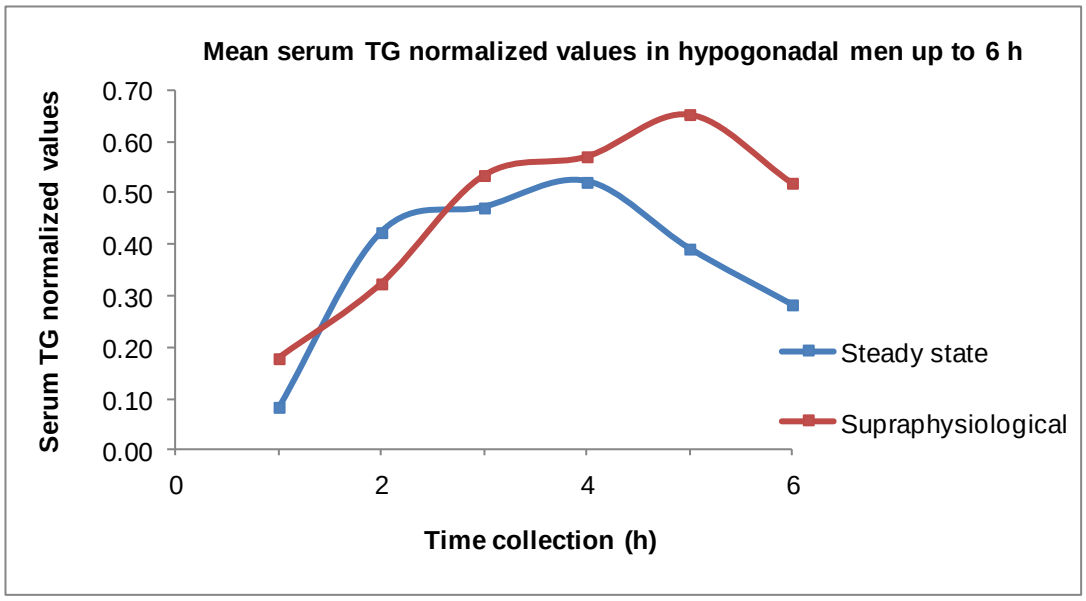


Figure 6.13 Serum TG normalized concentrations in hypogonadal men, up to 6 h, after 8 units of alcohol

The raw data for serum TG may be found in Figure 6.14.

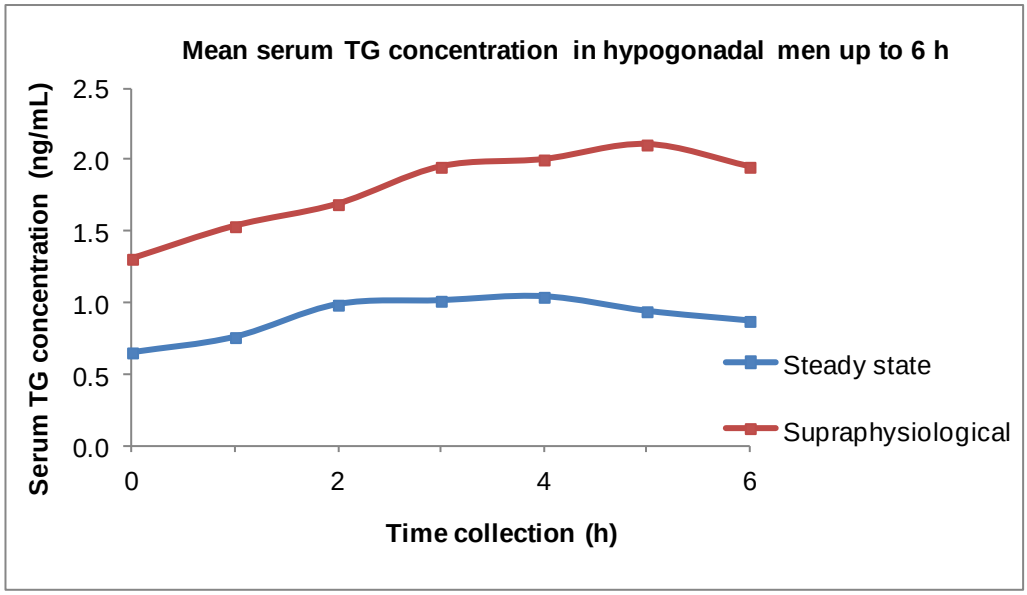


Figure 6.14 Serum TG raw concentrations in hypogonadal men, up to 6 h, after 8 units of alcohol

6.4 Discussion

In this chapter, after 8 units of alcohol, all the hypogonadal volunteers (who do not produce T) showed statistically significant increases in urinary T/E, T concentration, E concentration and 5α-DHT concentration. In Chapter 3, the study with eugonadal men showed a statistically significant increase in T/E, with urinary T concentration significantly increasing after 8 units as well as urinary 5α-DHT. The increase in urinary T/E, from the pre-administration value, in hypogonadal men was in the order of 120 % (for both steady state and hypogonadal groups), and 70 % in eugonadal men.

There was a statistically significant effect on serum T, similar to what happened in eugonadal men. The serum T rapidly decreases and the concentrations remain below the baseline (pre-administration) for 3 h. The serum TG concentration increased significantly over time.

Androstenedione did not show a statistically significant difference in concentration.

As the testicular steroidogenesis in hypogonadal men is considerably diminished, any influence of alcohol administration will be on elimination (metabolism and/or excretion) of T, the data not being confounded by an effect on gonadal production. It may be tentatively concluded that the metabolism of testosterone to androstenedione (the major intermediate in the metabolism of testosterone to the tetrahydro-17-oxo steroids, androsterone and etiocholanolone) is a rate-limiting step, such that a reduction in the activity of the oxidoreductase 17βHSD2, is not sufficient to have much of a consequence on the pool of circulating T, either in eugonadal men or hypogonadal men receiving replacement therapy. In other words, any decrease in the phase 1 metabolism of T due to alcohol, which would result in a raised serum T, is masked by the large pool of circulating T.

The increase in serum TG is a novel finding being unexpected, indicating an increase in phase 2 metabolism of T, although further investigations are required to prove this increase in the rate of conjugation.

Thus, the hypothesis that alcohol has an effect on hepatic metabolism of T (clearance) is supported by the data.

Chapter 7 CONCLUSIONS AND FURTHER WORK

The population's urinary T/E shows a bimodal distribution, with the modal values being 1 and 0.2. The latter thought to be caused by the gene polymorphism UGT2B17 *del/del* that codes an isoform of glucuronyl transferase, which is largely responsible for the conjugation testosterone.

Whether the majority or minority, the intra-individual variation in the T/E ratio in non-doped athletes is normally very small, but it is augmented by doping with testosterone or its precursors, e.g. androstenedione. The current WADA Technical Document (TD2004EAAS), states:

'In males, the individual T/E values have been shown to vary from their mean value by less than 30% (screening values). In females, a low concentration of some urinary steroids such as epitestosterone and testosterone, close to the limit of detection using current analytical methods occurs. Normal variation of up to 60% may be expected.'

For T/E to provide reliable evidence of doping, it is highly desirable that no permitted factor increases the T/E ratio to a value that would exceed the upper limit expected with intra-individual variation.

The suspicion that alcohol, which is not a prohibited substance in sports, increases this ratio had to be further investigated.

There is limited evidence in literature that alcohol could affect urinary T/E and thus this study aimed to better understand those mechanism(s) (Karila et al., 1996, Falk et al., 1988, Seppenwoolde-Waasdorp et al., 1996, Geyer et al., 1996, Mareck-Engelke et al., 1996).

Due to the possibility of physiological outliers, the definitive technique employed is GC-C-IRMS. However, it is still possible to obtain a false negative result with exogenous preparations of T exhibiting the same isotope signature than endogenous testosterone.

Furthermore, this technique is costly because of the instrumentation required and the skilled staff needed to operate it.

Our first question was if alcohol in serum could reach a concentration that would be sufficient to displace T from its binding proteins such that free serum T is raised, which would be then available for conjugation and urinary excretion, resulting in a raised urinary T/E ratio. An *in-vitro*

study, with serum samples from 10 volunteers was performed to test this hypothesis, using a concentration of alcohol equivalent to 700 mg%, but no increase in free serum T was found.

To better establish the origins of a urinary T/E increase after alcohol administration, two different doses of alcohol (4 and 8 units) were administered in different occasions to 10 eugonadal females and males. This was approximately 0.5 g/kg of alcohol for the 4 units, and 1 g/kg for 8 units. The highest dose of alcohol, 8 units, was administered to 3 hypogonadal men in two different occasions.

These occasions were one within a week after having had their Nebido® injection (hormone replacement therapy) where their serum T concentration is expected to be higher than the upper limit in eugonadal men (10 ng/mL) and were called 'supraphysiological phase'; and another occasion over one week after having had their injection, where it is expected that their serum T concentrations would be within the eugonadal range, named 'steady state phase'.

In females and males, after 4 and 8 units, there was a statistically significant increase in T/E. None of the eugonadal males went above the WADA threshold of 4. However, in females, after 8 units, three went above the WADA threshold and 6 above the 97.5^{th} percentile (2.65).

Overall, after normalization to pre-administration value, the mean for females showed an increase in T/E from baseline of 420 % after 8 units, and 190 % after 4 units. In the eugonadal males, the T/E increased 73 % from baseline after 8 units, and 36 % after 4 units.

For convenience of the reader, the relevant graphical data presented in previous chapters pertaining to the conclusions may be found at the end of this chapter.

There was a statistically significant effect in urinary T concentration, E concentration and in 5α-DHT urinary concentrations as well.

After 8 units, female volunteers urinary T concentration increased 217 % when compared to baseline, and after 4 units it increased 172 %. In eugonadal males, the urinary T concentration increased by 52 % after 8 units, and 32 % after 4 units.

The urinary E concentration showed a significant decrease in both females and males. Females decreased 55 % after 8 units, and 36 % after 4 units; whereas males showed a more modest decrease at 32 % after 8 units, and 22 % after 4 units.

The fact that the relative increase in urinary T concentration in females is considerably higher than in the eugonadal males, combined with the larger relative decrease in the urinary E concentration, explains the higher increase in T/E when compared to males, where none went above the WADA threshold of 4. Therefore, the hypothesis that two different doses of alcohol ingested within an hour in two different occasions would influence urinary T/E in females and males due to an increase in excretion was not rejected, although ethanol ingestion has much more serious implications for female athletes, as the T/E threshold can be exceeded.

Notwithstanding, in both male and female athletes, alcohol ingestion can cause sufficient variation to the T/E ratio to abnormally affect the intra-individual profile. Some athletes who may know, or get to learn of this influence, may consume alcohol deliberately, in advance of the short-notice of out-of-competition testing, to mask their use of testosterone.

The analyst should consider that this is a possiblity and must use GC-C-IRMS in an attempt to ascertain whether such a manipulation has occurred. Conversely, with a GC-C-IRMS finding that is unremarkable, i.e., no hint of steroid administration, it would be fitting to conclude that alcohol ingestion was the innocent cause and that T/E result should not be included in future assessment's of that individual's T/E profile.

The mechanism of the large increase in the T/E ratio in females may be related to the fact that alcohol increases the ratio of NADH/NAD^+, which in turn may suppress the oxidation of T into AD, thus increasing the concentration of T.

The increases in serum T in females are in agreement with decreases in serum LH (although these were not significant) and a significant decrease in urinary LH over time. The current investigation supported this supposition, as there was a significant increase in T, the relative increase being 60 % after 8 units, and a rapid decrease in AD in females (40 %).

The fact that this study only showed a very modest increase in serum T in eugonadal males (2 %), following an initial dip of approximately 20 %, when compared to females does not mean

that there is a lack of inhibition of hepatic oxidation of testosterone in men, but rather that this effect is obscured by the larger pool of testosterone present.

In eugonadal women, the pool of circulating T is considerably lower, the serum concentration being approximately a tenth of that found in eugonadal men, and if the enzymatic oxidation of T to androstenedione is not saturated, the impact of alcohol on this conversion is such that there is a relatively large increase in the serum T concentration (approximately 5-fold), which is reflected in a relatively large increase in the urinary T concentrations and the urinary T/E ratio. In men, 17β-HSD2 may be saturated with testosterone, and so even with inhibition of its activity by ethanol, the amount of T that would have been otherwise converted to androstenedione, would be relatively small to the amount of testosterone circulating, i.e. an increase in the concentration of serum T would be expected, but this would be relatively small to that in females. Indeed, the increase in serum T was only 2 % at 4 h, the time when the T/E ratio was maximising, nothing like the ~5-fold increase in serum T observed in the women at that time.

The decrease in the urinary E concentrations in the women may also be rationalised by the effect on inhibition of activity of 17β-HSD2. Just as there will be an increase in serum T, there should be a decrease in androstenedione and this was the case. One of the precursors of E formation is androstenedione, and it is likely that any hepatic conversion of androstenedione will be to E, which will then be available for phase 2 metabolism as EG, (which after hydrolysis for urinary analysis is treated as E). Although androstenedione is probably the minor of the two biosynthetic precursors of epitestosterone, the major one being DHEA, it is a precursor nonetheless (Starka, 2003). Thus a fall in hepatic androstenedione production would be expected to result in a modest fall in E glucuronide production, explaining the modest decrease in urinary E following alcohol administration.

In eugonadal men, there was a similar decrease in the concentration of serum AD and there was a minor decrease in urinary E concentration, which may also explain the modest decrease in urinary E in man.

If serum T is decreasing following an increase in metabolism thus increasing its phase 2 metabolite, TG, it is also possible that E, which follows the same excretion pathway as T, is being metabolized into EG (its phase 2 metabolite).

The study with hypogonadal men on hormone replacement therapy, where gonadal production of T has been eliminated as a contributing factor, shed some light on our previous findings.

This part of the research study, showed a statistically significant effect in urinary T/E, urinary T, E and 5α-DHT concentrations; and serum T. No significant effect was shown in AD concentration.

The steady state group had an increase in urinary T/E from its pre-administration value of 120 % after 3 h, whereas the supraphysiological group had an increase of 130 % from their pre-administration value after 5 h. With the eugonadal volunteers, after 8 units, the increase from their pre-administration value was approximately 70 % after 2 h.

The significant increase in urinary T/E was consistent with a significant urinary T increase, of 134 % in supraphysiological volunteers and 50 % in steady state. The urinary E concentration decreased 19 % from their baseline values in volunteers in their supraphysiological state and increased in steady state volunteers by 56 %.

The fact that there is a higher increase in T/E in supraphysiological men is consistent with a higher increase in urinary T concentration in supraphysiological volunteers together with a decreased urinary E concentration, when compared to steady state volunteers.

These increases were followed by suppression in serum T in the first few hours after alcohol administration, which could be indicative that the increase in urinary T/E is related to hepatic clearance of T and/or renal excretion.

The serum concentration of TG, a phase 2 metabolite of T, was measured in hypogonadal men. In eugonadal volunteers this was not possible since approximately 50 % of the samples were below the LLOQ.

Therefore, our final hypotheses, that alcohol has an effect on hepatic clearance of T has not been rejected.

To reiterate, the increase in urinary T/E in eugonadal and hypogonadal males follows an increase in urinary total concentrations (aglycone plus free fraction) of T and E. With alcohol acting as a diuretic, the urinary flow rate increases, thus increasing glomerular filtration in the

kidneys and urinary TG concentration would be likely to increase. The serum T concentration decreased and serum TG increased (only measurable in hypogonadal volunteers), indicative of an increase in metabolism.

In females, the increase in urinary T/E is linked to the high urinary T concentration, dissociated from any increase in urinary E concentration. This is most likely a reflection of the increase in serum T, due to the suppression in the activity of the enzyme 17β-HSD2, thus shifting the conversion of T into AD, towards T.

The increase in serum T in females in this research study was not as accentuated as in the study by Sarkola *et al.*(Sarkola et al., 2001). The differences observed in serum T may be related to the analytical technique employed, where the T concentrations were measured via immunoassay which may have cross-reacted with TG, thus increasing the serum T concentration. With mass-spectrometry, the technique used in our research study, such does not happen.

Analysis of serum LH concentration showed a decrease in females after 4 and after 8 units, and a decrease in males after 4 units and an increase after 8 units but these results were not statistically significant. These results contradict those of Karila *et al.*, where a serum increase in LH was verified after 2 g/kg of alcohol, even though not significant (Karila et al., 1996). The study performed by Karila *et al.* was done in 4 females and 4 males, with serum collection 24h and 48 h after alcohol administration which may have introduced variability in the results.

Urinary LH concentration had a significant decrease in females over time, and any changes in males were not significant. Due to its negative feedback mechanism, a decrease in LH would be in agreement with an increase in serum T, which was verified in females.

Thus the hypothesis that any increase in urinary T concentration is due to an increase in LH was discounted.

In this study, we found that both females and males had an increased T/E when compared to their baseline values, with females showing an evidential increase afer 8 units. Our study had a different experiment design when compared to previous published studies (Falk et al., 1988, Karila et al., 1996, Seppenwoolde-Waasdorp et al., 1996, Mareck-Engelke et al., 1996).

Our study shows consistency. It was performed with a bigger population (n=10 for females and n=9 for males) decreasing inter-individual variability, urine and blood were collected hourly which aided in the understanding of the clearance (metabolic and/or excretion) of T and E and their conjugates and gives more reliability to the statistical treatment. Our study also recruited hypogonadal men on hormone replacement therapy to exclude the possiblity of gonadal production of T influence the urinary T/E ratio due to alcohol.

Concerning further work, it would be interesting to perform this study in a bigger population and with smaller collection windows so as to be able to perform an appropriate correlation between serum T and AD, serum T and urinary T/E, and both of these with the blood alcohol concentration.

Regarding EtG urinary concentration, perhaps this test ought to be included in the athlete's biological passport to help prevent an 'atypical finding' from flagging an athlete. Although it does not seem possible to establish a universal threshold due to individual variation, it could be fruitful nevertheless, to perform this type of study in athletes so that at least there could be an individual threshold established.

One way of testing the hypothesis that the increase in urinary T/E after alcohol administration is due an increase in metabolism and/or renal excretion, would be to administer stable labelled TG and T, and verify any changes in clearance (hepatic and/or renal).

In summary, females are overall more affected by alcohol than men with its effects being translated into an increase in urinary T/E that exceed the WADA threshold. In men, there is also a significant increase but with the amount of alcohol administered, it was not enough to go above the threshold.

Our data seems to indicate that the increase in urinary T/E in males is either due to an increase in hepatic clearance or an increase in the excretion of T, not to an increase in production; and in females most likely due to a suppression of T metabolism, which will then increase circulating serum T concentration and hence increase the urinary T/E ratio.

Due to the different production sites of T, women seem to have their urinary T/E affected in a more acute way.

This makes an interesting correlation with the poem from the American writer Ogden Nash, in 'Reflections on Ice-Breaking':

Candy

Is dandy

But liquor

Is quicker.

7.1 Eugonadal males, normalized graphical data

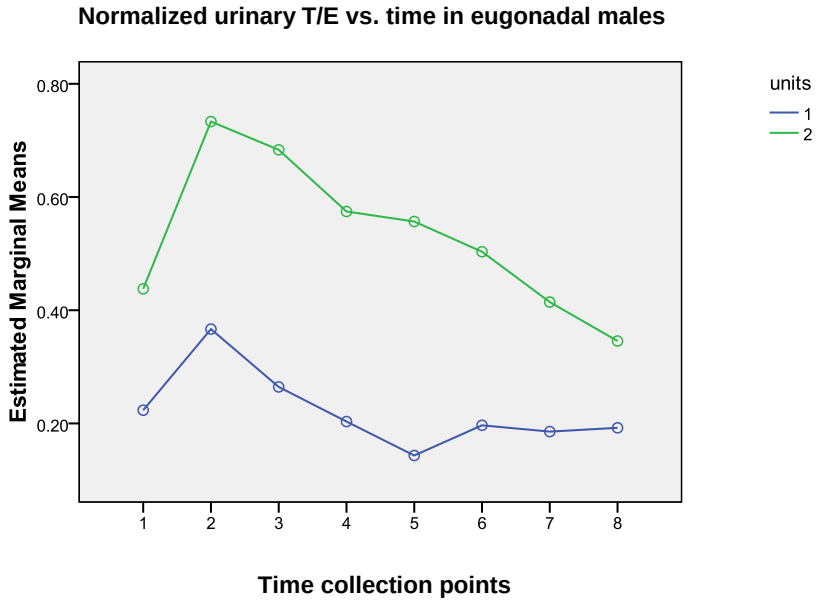


Figure 7.1 Normalized T/E in males. Units 1 = 4 units, 2 = 8 units

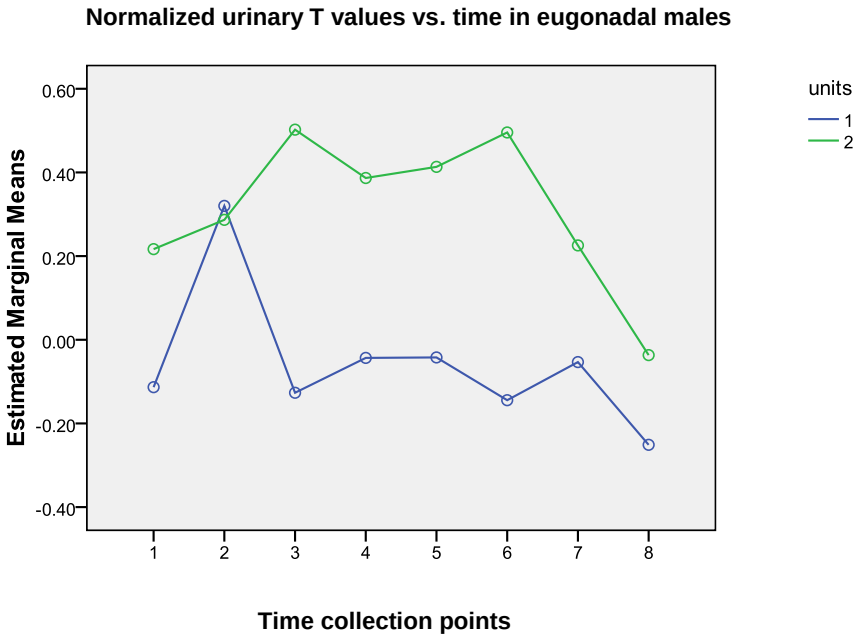


Figure 7.2 Normalized urinary T values in males. Units 1 = 4 units, 2 = 8 units

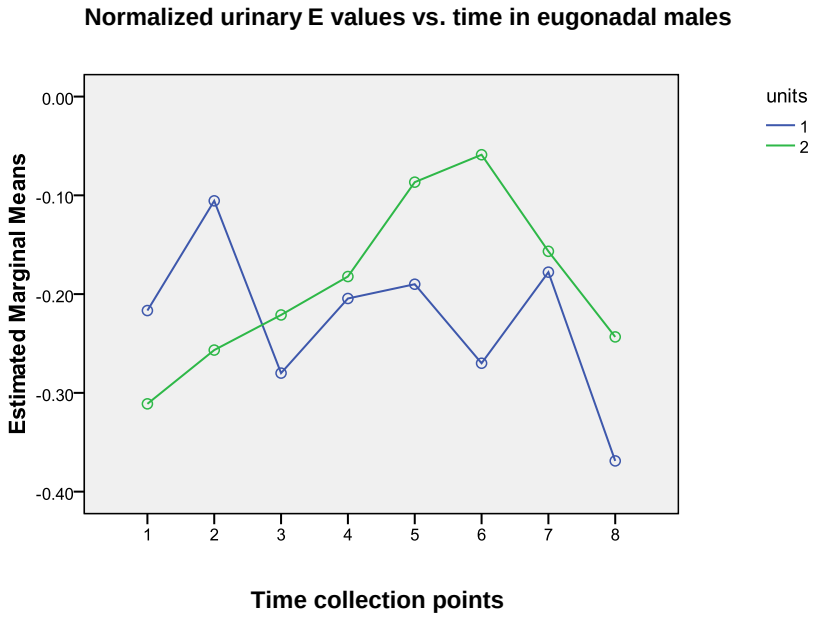


Figure 7.3 Normalized urinary E values in males. Units 1 = 4 units, 2 = 8 units

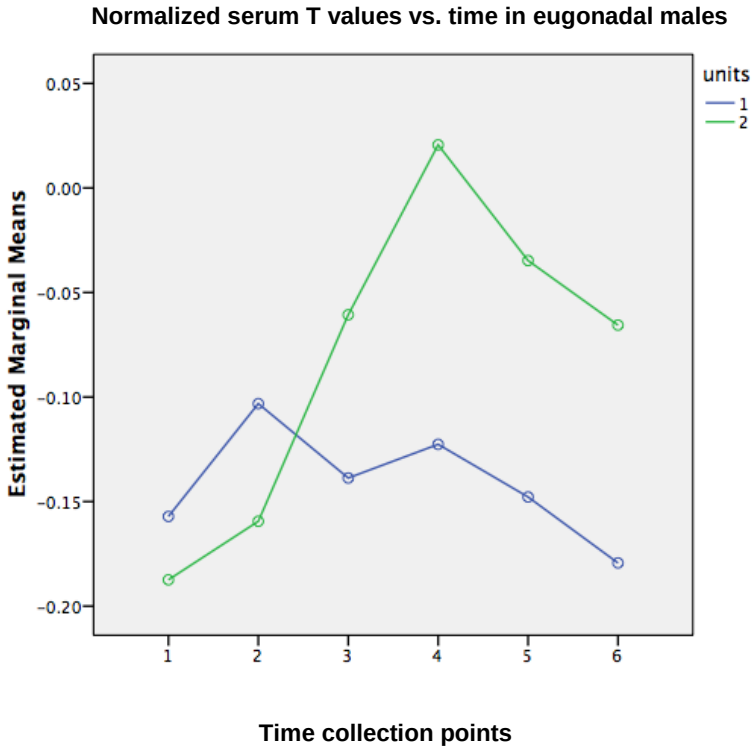


Figure 7.4 Normalized serum T values in eugonadal men. Units 1 = 4 units, 2 = 8 units

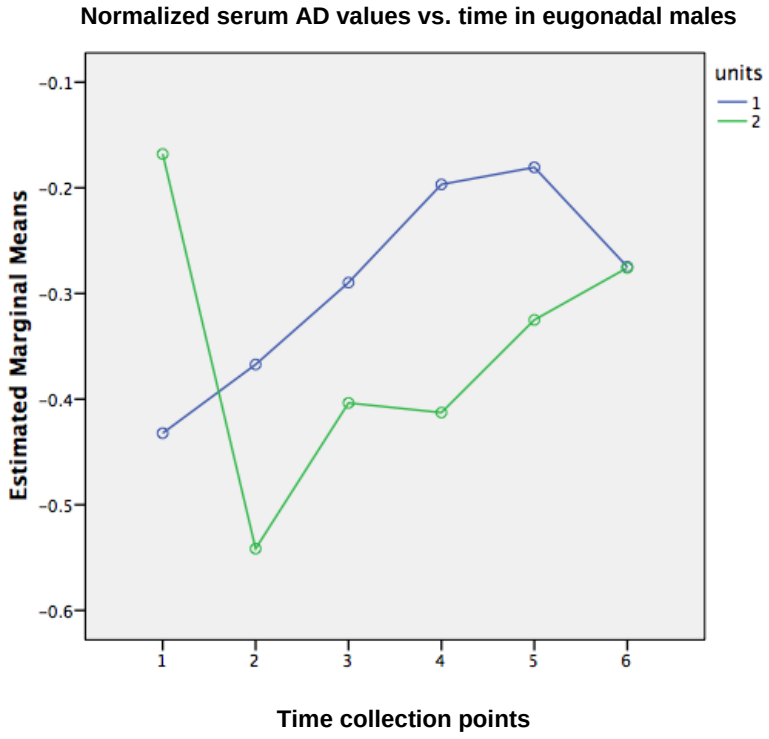


Figure 7.5 Normalized serum AD values in eugonadal men. Units 1 = 4 units, 2 = 8 units

7.2 Eugonadal females, normalized graphical data

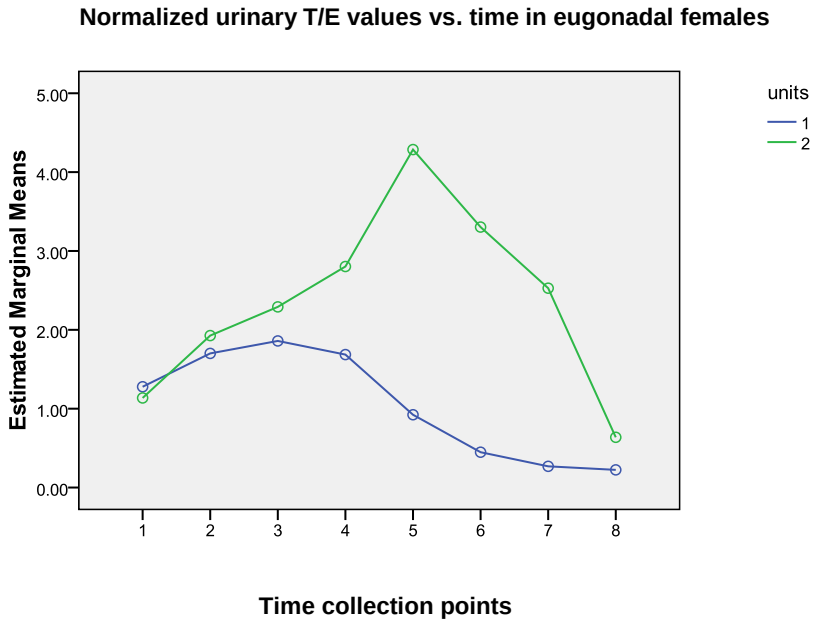


Figure 7.6 Normalized T/E in females. Units 1 = 4 units, 2 = 8 units

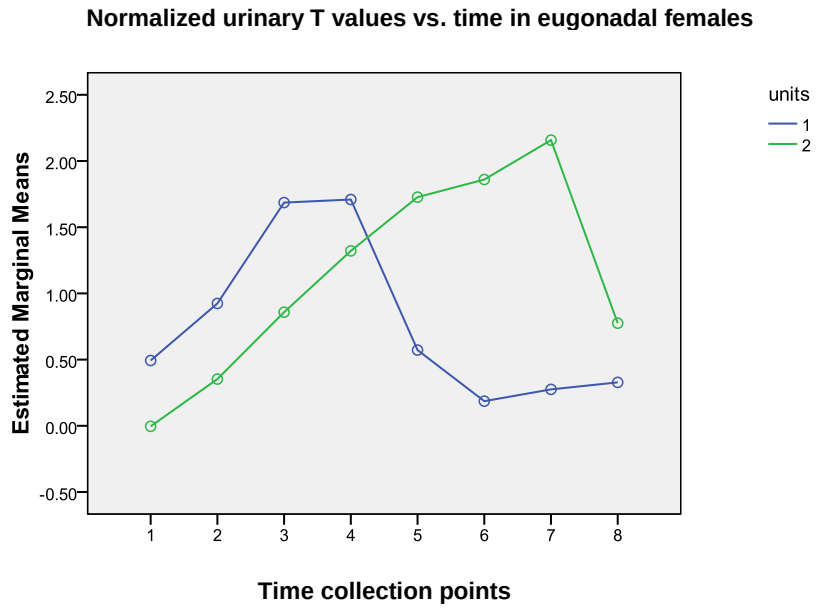


Figure 7.7 Normalized urinary T values in females. Units 1 = 4 units, 2 = 8 units

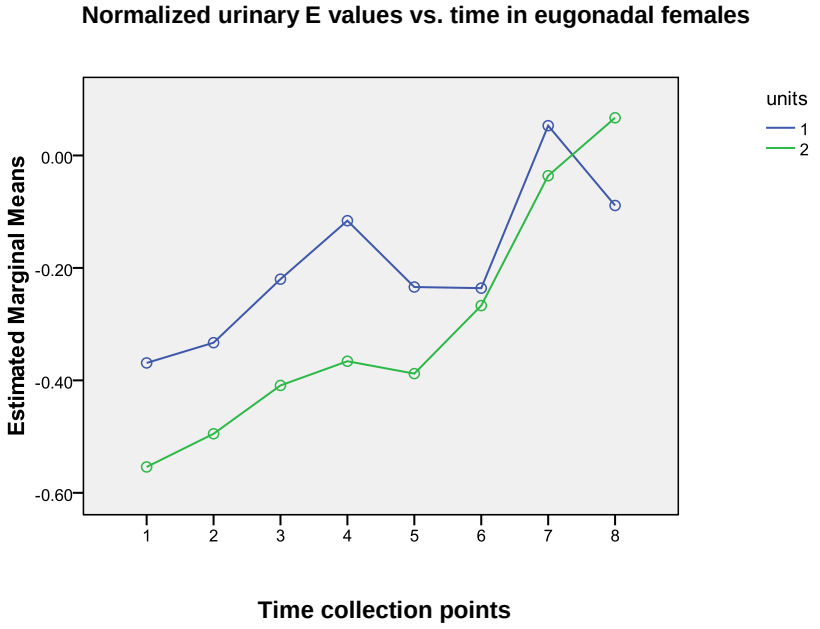


Figure 7.8 Normalized urinary E values in females. Units 1 = 4 units, 2 = 8 units

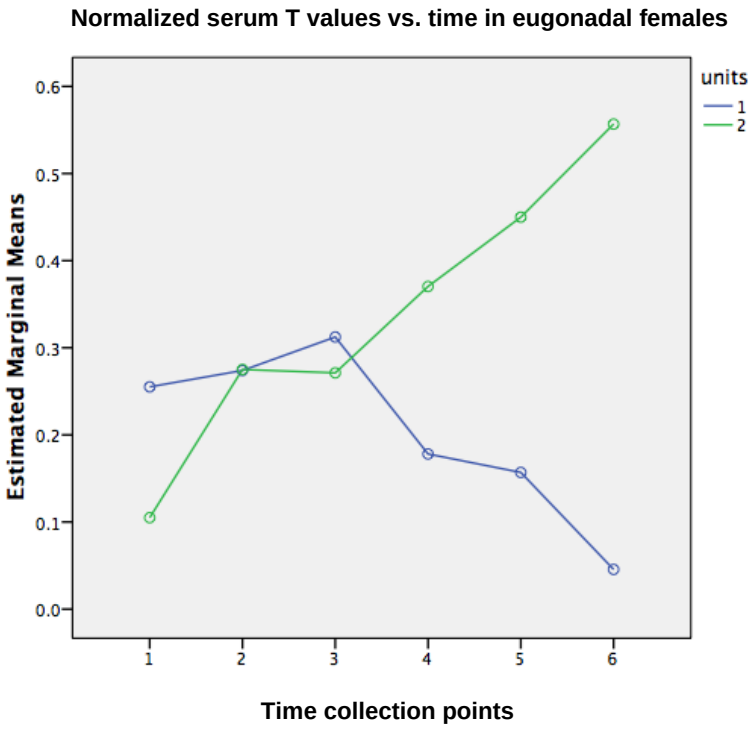


Figure 7.9 Normalized serum T values for eugonadal females. Units 1 = 4 units, 2 = 8 units

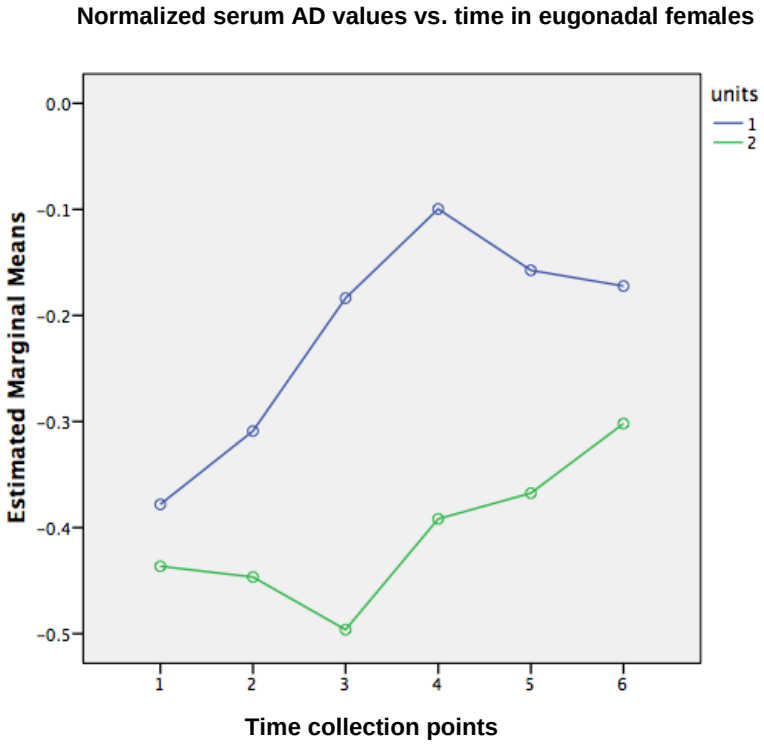


Figure 7.10 Normalized serum AD values in eugonadal females. Units 1 = 4 units, 2 = 8 units

7.3 Hypogonadal men, normalized graphical data

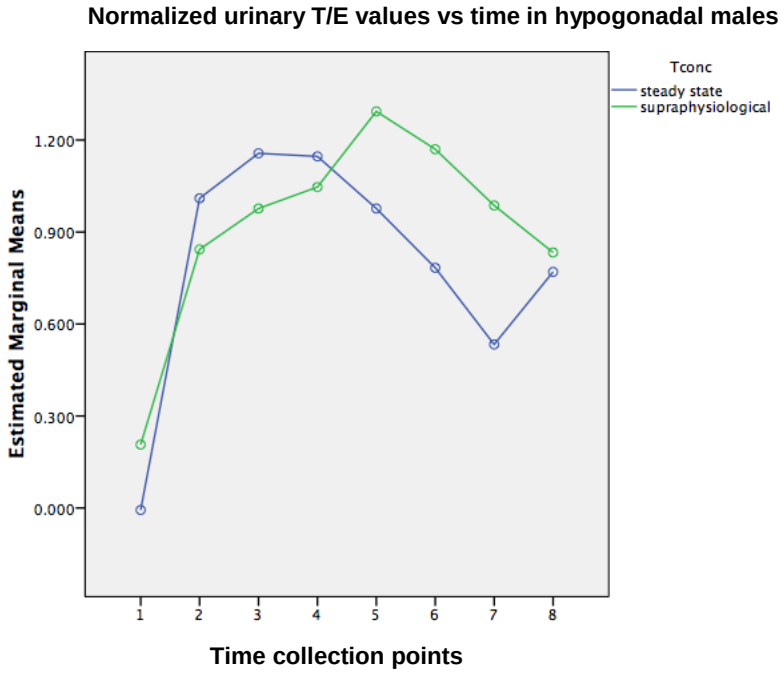


Figure 7.11 Normalized urinary T/E in hypogonadal men after 8 units of alcohol

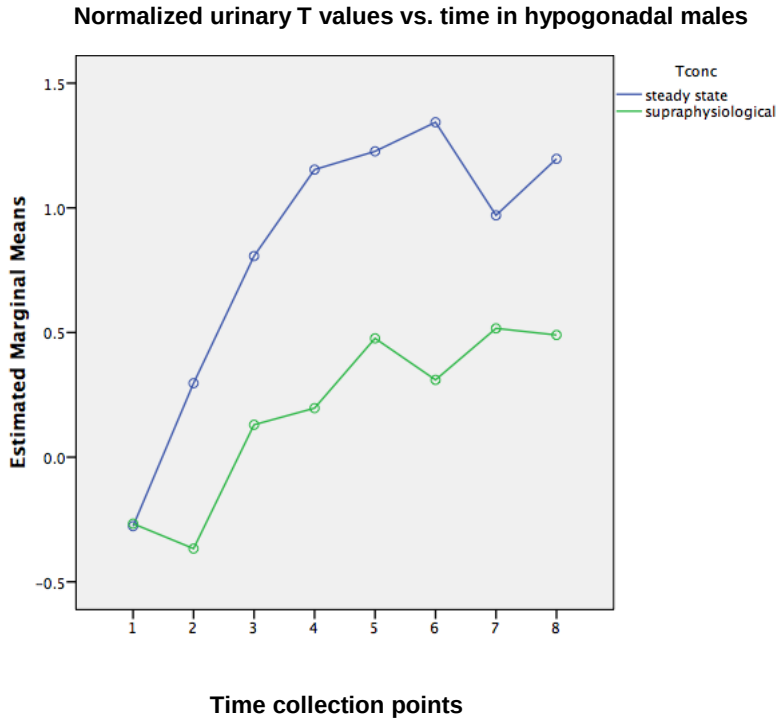


Figure 7.12 Normalized urinary T values in hypogonadal men after 8 units of alcohol

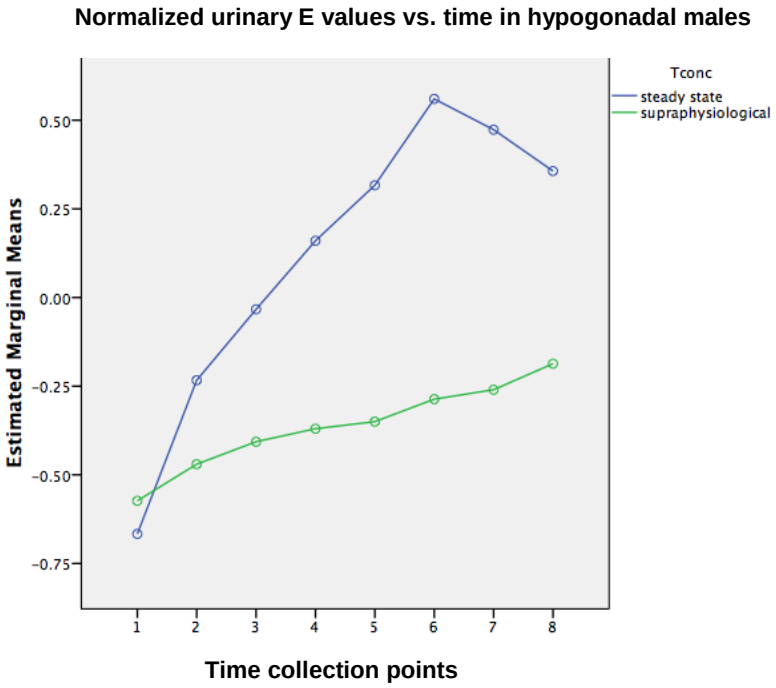


Figure 7.13 Normalized urinary E values in hypogonadal men after 8 units of alcohol

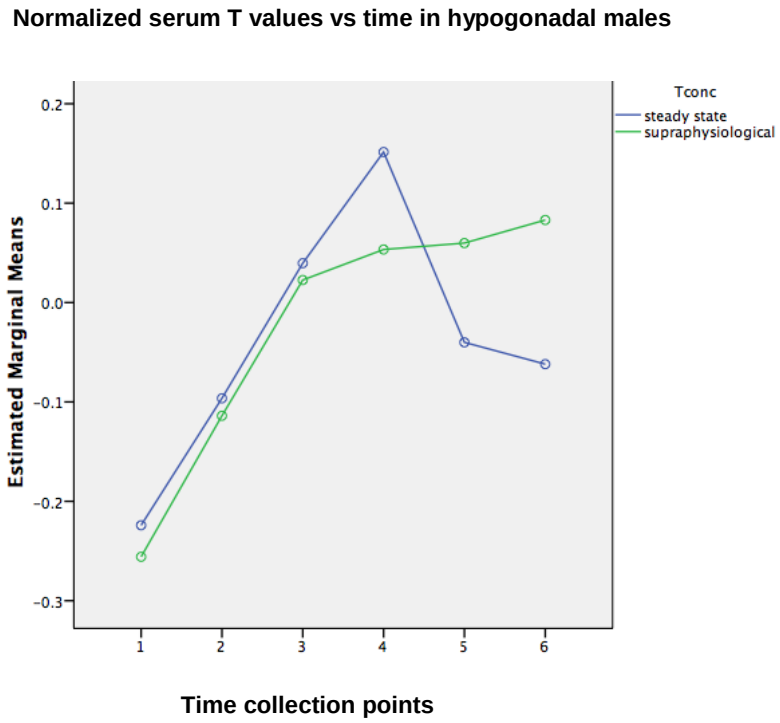


Figure 7.14 Normalized serum T values in hypogonadal men after 8 units of alcohol

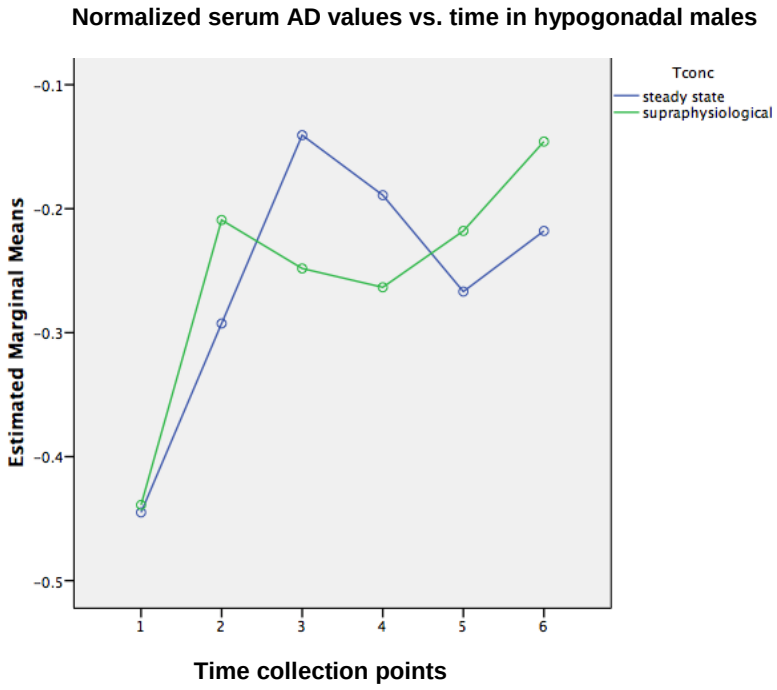


Figure 7.15 Normalized serum AD values in hypogonadal men after 8 units of alcohol

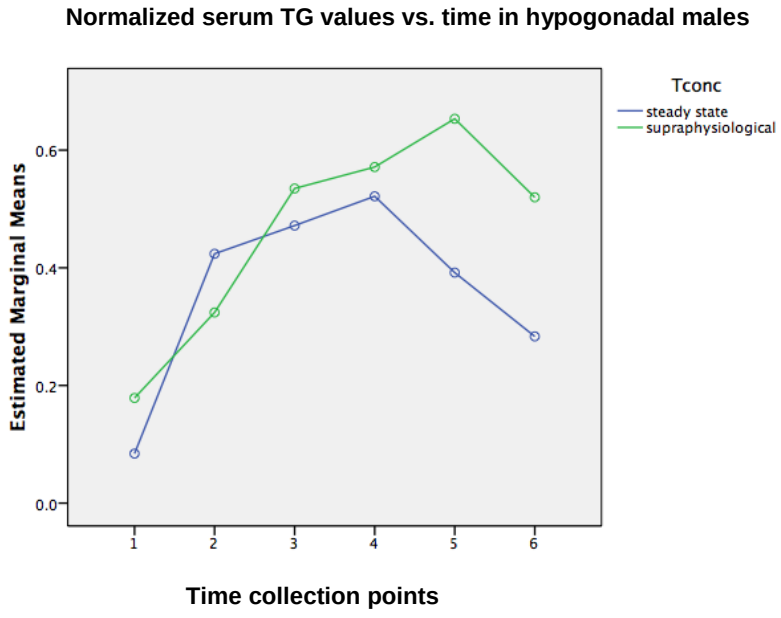


Figure 7.16 Normalized serum TG values in hypogonadal men after 8 units of alcohol

Chapter 8 APPENDICES

8.1 Endocrine glands

Appendix 8.1 Endocrine glands

Endocrine gland	Function	Secreting hormones
Brain (including hypothalamus)	• Controls nervous system but is one of the most important endocrine gland • Specialized nerve cells in hypothalamus responsible for hormone synthesis • Hormones are transported along the axon to the nerve terminal where they are released into the portal blood system, that carries them to the pituitary gland, just beneath the hypothalamus • These hormones control salt and water balance, sexual function and behaviour, lactation and the body's response to stress	• Corticotropin-releasing hormone (CRH) or factor (CRF) • Dopamine (prolactin-inhibiting hormone – PIF) • Growth-hormone releasing hormone (GRH) • Gonadotropin-releasing hormone (GnRH, LHRH) • Somatostatin (growth-hormone-inhibiting hormone; GHIH) • Tyrotopin-releasing hormone (TRH) • Oxytocin • Vasopressin
Pituitary gland	• Synthesis of hormones that control many of the other endocrine glands • Composed of different lobes (anterior, posterior and intermediate) that are responsible for hormone synthesis	<u>Anterior:</u> • Corticotropin (adrenocorticotropic hormone-ACTH) • FSH • Luteinizing hormone (LH) • Prolactin • Thyrotropin (thyroid-stimulating hormone – TSH) • Growth hormone (GH) <u>Posterior:</u> • Oxytocin

		• Vasopressin Intermediate • Melanocyte-stimulating hormone (MSH)
Thyroid gland	• In humans, it adheres to, and is located in front of the trachea • Iodine is used for the synthesis of thyroid hormones. Low levels of iodine cause hyper- and hypothyroidism	• Thyroxine (T_4) • Tri-iodothyronine (T_3)
Parathyroid gland	• Integrated in thyroid gland • Its hormone is responsible for controlling levels of calcium and phosphate in the blood	• Parathyroid hormone (parathormone, PTH)
Parafollicular cells	• In thyroid's follicles	• Calcitonin
Adrenal glands	• Situated above the kidneys • Composed of cortex (outer layer) and medulla (inner layer) • Cortex produces steroid hormones • Medulla produces catecholamines	<u>Cortex:</u> • Glucocorticoids (eg. cortisol) • Mineralocorticoids (eg. aldosterone) • Sex hormones (eg. androstenedione) <u>Medulla:</u> • Adrenaline • Noradrenaline
Endocrine[11] pancreas	• Cells spread around the larger exocrine pancreas (adjacent to stomach)	• Insulin • Glucagon • Somatostatin • Pancreatic polypeptide

[11] An endocrine gland is a gland that produces hormones and secretes them directly into the blood stream rather than through a duct (eg. adrenal glands, pituitary gland, testes, ovaries and the thyroid gland). An exocrine gland secretes its products (not known as hormones) into ducts which lead to the outside environment (e.g. sweat glands, salivary glands and mammary glands).

Ovary	• Major female reproductive gland	• Estrogen • Progesterone • Relaxin
Testis	• Major male reproductive gland	• Testosterone • Inhibin • Müllerian regression factor (inhibiting fetus)
Gastrointestinal tract (GIT)	• Largest endocrine organ • Produces several autocrine, paracrine and endocrine hormones [12]	• Cholecystokinin (CCK) • Gastric inhibitory peptide (GIP) • Gastrin • Neurotensin • Secretin • Substance P • Vasoactive intestinal peptide (VIP)
Pineal gland	• Situated in brain • Involved in rhythms, such as reproductive rhythms of animals which breed seasonally • Role in humans still unknown	• Melatonin
Kidney	• Filters soluble waste products and drugs from circulation • Produces hormones involved in the control of blood pressure and erythropoiesis (production of red blood cells)	• Renin • Erythropoietin
Placenta	• Organ of pregnancy serving the developing fetus	• Chorionic gonadotropin (CG, hCG – h for human)
Thymus	• Essential for normal fetal growth • Retains immature lymphocytes and processes them to a mature state, crucial for the immune system	• Thymopoietin • Thymulin

[12] Endocrine hormones are the ones that are secreted into the blood and are carried by it to the cells they will act on, autocrine hormones act on the secretory cell itself and paracrine act on contiguous cells PORTERFIELD, S. P. 2001a. Chapter 1 - Introduction to the endocrine system. *Endocrine physiology.* 2nd ed. Missouri: Mosby.

8.2 Intra-assay variability for calibrants 0.15, 0.30 and 2.5 ng/mL

Appendix 8.2 Intra-assay variability for calibrants 0.15, 0.30 and 2.5 ng/mL

Compound : Testosterone **Conc. units: ng/mL**

Drug Conc.	Drug Response	Internal Std. Response	Response Ratio	Percentage Error
0.00	17.00	89.00	0.191	
0.10	103.00	81.00	1.272	-11.55
0.25	167.00	78.00	2.141	-14.22
0.50	695.00	144.00	4.826	20.72
1.00	721.00	90.00	8.011	6.51
2.00	1198.00	85.00	14.094	-2.67

REGRESSION STATISTICS

Correlation Coeff.	**0.9958**	**Percentage Fit**	**99.17**
Adjusted R Square	0.9896	Std. Deviation	0.5330
xmean	0.6417	No. Observations	6
ymean	5.0892	X-Intercept	-0.0958

	Deg.of Freedom	Sum of Squares	Mean Squares	F Ratio
Regression	1	135.8170	135.8170	478
Residual	4	1.1362	0.2840	
Total	5	136.9532	27.3906	

	Coefficients	Std. Err. Estimates
Y-Intercept	0.6612	0.2972
Slope	6.9007	0.3156

Expected dialysate T of 0.15 ng/mL

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV034	63.00	62.00	1.016	0.05	
JLV034	77.00	59.00	1.305	0.09	
JLV034	67.00	57.00	1.175	0.07	
No.of Samples	3	**Mean**	1.17	**0.07**	**0.06**

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV035	98.00	72.00	1.361	0.10	
JLV035	71.00	60.00	1.183	0.08	
JLV035	65.00	51.00	1.275	0.09	
No.of Samples	3	**Mean**	1.27	**0.09**	**0.06**

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV036	94.00	72.00	1.306	0.09	
JLV036	97.00	82.00	1.183	0.08	
JLV036	92.00	76.00	1.211	0.08	
No.of Samples	3	**Mean**	1.23	**0.08**	**0.06**

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV037	89.00	66.00	1.348	0.10	
JLV037	64.00	55.00	1.164	0.07	
No.of Samples	2	**Mean**	1.26	**0.09**	**0.07**

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV038	124.00	99.00	1.253	0.09	
JLV038	100.00	72.00	1.389	0.11	
JLV038	106.00	91.00	1.165	0.07	
No.of Samples	3	**Mean**	1.27	**0.09**	**0.06**

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV039	60.00	60.00	1.000	0.05	
JLV039	43.00	38.00	1.132	0.07	
JLV039	81.00	54.00	1.500	0.12	
No.of Samples	3	**Mean**	1.21	**0.08**	**0.06**

Expected dialysate T of 0.30 ng/mL

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV040	287.00	88.00	3.261	0.38	
JLV040	277.00	78.00	3.551	0.42	
JLV040	298.00	78.00	3.821	0.46	
No.of Samples	3	**Mean**	3.54	**0.42**	**0.06**

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV041	243.00	76.00	3.197	0.37	
JLV041	274.00	73.00	3.753	0.45	
JLV041	316.00	77.00	4.104	0.50	
No.of Samples	3	**Mean**	3.68	**0.44**	**0.06**

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV042	176.00	43.00	4.093	0.50	
JLV042	227.00	54.00	4.204	0.51	
JLV042	205.00	58.00	3.534	0.42	
No.of Samples	3	**Mean**	3.94	**0.48**	**0.06**

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV043	254.00	81.00	3.136	0.36	
JLV043	285.00	75.00	3.800	0.45	
JLV043	269.00	78.00	3.449	0.40	
No.of Samples	3	**Mean**	3.46	**0.41**	**0.06**

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV044	246.00	65.00	3.785	0.45	
JLV044	272.00	88.00	3.091	0.35	
JLV044	208.00	56.00	3.714	0.44	
No.of Samples	3	**Mean**	3.53	**0.42**	**0.06**

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV045	241.00	67.00	3.597	0.43	
JLV045	296.00	74.00	4.000	0.48	
JLV045	285.00	75.00	3.800	0.45	
No.of Samples	3	**Mean**	3.80	**0.45**	**0.06**

Expected T dialysate of 2.5 ng/mL

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV046	3099.00	149.00	20.799	2.92	
JLV046	2329.00	117.00	19.906	2.79	
JLV046	2838.00	129.00	22.000	3.09	
No.of Samples	3	**Mean**	20.90	**2.93**	**0.12**
Sample Name	Drug Response	Std. Response	Response Ratio	**Drug Conc.**	**Std.Err.**
JLV047	2356.00	106.00	22.226	3.13	
JLV047	2282.00	107.00	21.327	2.99	
JLV047	1865.00	93.00	20.054	2.81	
No.of Samples	3	**Mean**	21.20	**2.98**	**0.12**
Sample Name	Drug Response	Std. Response	Response Ratio	**Drug Conc.**	**Std.Err.**
JLV048	2464.00	127.00	19.402	2.72	
JLV048	3057.00	150.00	20.380	2.86	
JLV048	2594.00	129.00	20.109	2.82	
No.of Samples	3	**Mean**	19.96	**2.80**	**0.11**
Sample Name	Drug Response	Std. Response	Response Ratio	**Drug Conc.**	**Std.Err.**
JLV049	3190.00	158.00	20.190	2.83	
JLV049	3753.00	170.00	22.076	3.10	
JLV049	3565.00	159.00	22.421	3.15	
No.of Samples	3	**Mean**	21.56	**3.03**	**0.12**
Sample Name	Drug Response	Std. Response	Response Ratio	**Drug Conc.**	**Std.Err.**
JLV050	3366.00	147.00	22.898	3.22	
JLV050	3059.00	141.00	21.695	3.05	
JLV050	2975.00	135.00	22.037	3.10	
No.of Samples	3	**Mean**	22.21	**3.12**	**0.13**
Sample Name	Drug Response	Std. Response	Response Ratio	**Drug Conc.**	**Std.Err.**
JLV051	2628.00	142.00	18.507	2.59	
JLV051	3099.00	120.00	25.825	3.65	
JLV051	2756.00	129.00	21.364	3.00	
No.of Samples	3	**Mean**	21.90	**3.08**	**0.12**

8.3 Testosterone in aqueous solution

Appendix 8.3 Testosterone in aqueous solution

Compound : Testosterone **Conc. units: ng/mL**

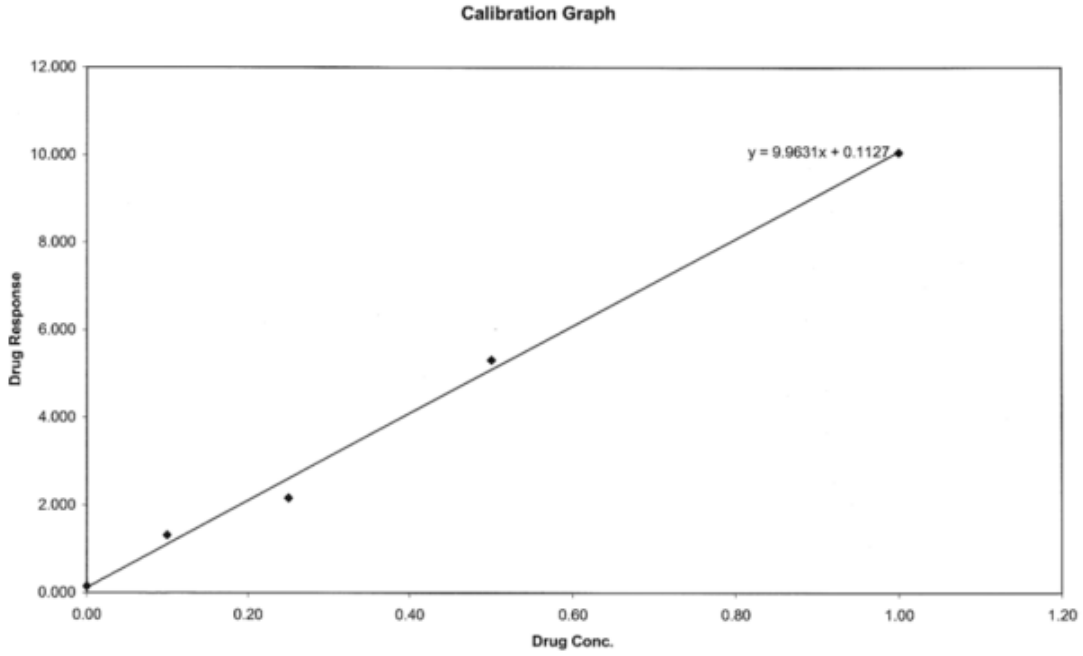
Drug Conc.	Drug Response	Internal Std. Response	Response Ratio	Percentage Error
0.00	9.00	60.00	0.150	
0.10	58.00	44.00	1.318	21.00
0.25	231.00	107.00	2.159	-17.85
0.50	531.00	100.00	5.310	4.33
1.00	865.00	86.00	10.058	-0.18

REGRESSION STATISTICS

Correlation Coeff.	**0.9977**	**Percentage Fit**	**99.54**
Adjusted R Square	0.9939	Std. Deviation	0.3107
xmean	0.3700	No. Observations	5
ymean	3.7990	X-Intercept	-0.0113

	Deg.of Freedom	Sum of Squares	Mean Squares	F Ratio
Regression	1	63.3299	63.3299	656
Residual	3	0.2897	0.0966	
Total	4	63.6196	15.9049	

	Coefficients	Std. Err. Estimates
Y-Intercept	0.1127	0.2001
Slope	9.9631	0.3890



Expected dialysate T of 0.15 ng/mL

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV 010	21.00	102.00	0.206	0.01	
JLV 010	14.00	76.00	0.184	0.01	
JLV 010	11.00	71.00	0.155	0.00	
No.of Samples	3	**Mean**	0.18	**0.01**	**0.03**
Sample Name	Drug Response	Std. Response	Response Ratio	**Drug Conc.**	**Std.Err.**
JLV 011	90.00	86.00	1.047	0.09	
JLV 011	77.00	82.00	0.939	0.08	
JLV 011	73.00	83.00	0.880	0.08	
No.of Samples	3	**Mean**	0.96	**0.08**	**0.03**
Sample Name	Drug Response	Std. Response	Response Ratio	**Drug Conc.**	**Std.Err.**
JLV 012	98.00	100.00	0.980	0.09	
JLV 012	95.00	94.00	1.011	0.09	
JLV 012	97.00	94.00	1.032	0.09	
No.of Samples	3	**Mean**	1.01	**0.09**	**0.03**
Sample Name	Drug Response	Std. Response	Response Ratio	**Drug Conc.**	**Std.Err.**
JLV 013	53.00	50.00	1.060	0.10	
JLV 013	48.00	47.00	1.021	0.09	
JLV 013	47.00	43.00	1.093	0.10	
No.of Samples	3	**Mean**	1.06	**0.09**	**0.03**
Sample Name	Drug Response	Std. Response	Response Ratio	**Drug Conc.**	**Std.Err.**
JLV 014	36.00	42.00	0.857	0.07	
JLV 014	35.00	38.00	0.921	0.08	
JLV 014	35.00	35.00	1.000	0.09	
No.of Samples	3	**Mean**	0.93	**0.08**	**0.03**
Sample Name	Drug Response	Std. Response	Response Ratio	**Drug Conc.**	**Std.Err.**
JLV 015	89.00	82.00	1.085	0.10	
JLV 015	83.00	80.00	1.038	0.09	
JLV 015	76.00	72.00	1.056	0.09	
No.of Samples	3	**Mean**	1.06	**0.10**	**0.03**

Expected dialysate T of 0.30 ng/mL

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV 016	9.00	118.00	0.076	0.00	
JLV 016	8.00	112.00	0.071	0.00	
JLV 016	9.00	111.00	0.081	0.00	
No.of Samples	3	**Mean**	0.08	**0.00**	**0.03**

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV 017	382.00	149.00	2.564	0.25	
JLV 017	383.00	144.00	2.660	0.26	
JLV 017	350.00	131.00	2.672	0.26	
No.of Samples	3	**Mean**	2.63	**0.25**	**0.02**

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV 018	331.00	92.00	3.598	0.35	
JLV 018	309.00	92.00	3.359	0.33	
JLV 018	253.00	69.00	3.667	0.36	
No.of Samples	3	**Mean**	3.54	**0.34**	**0.02**

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV 019	348.00	119.00	2.924	0.28	
JLV 019	321.00	111.00	2.892	0.28	
JLV 019	316.00	107.00	2.953	0.29	
No.of Samples	3	**Mean**	2.92	**0.28**	**0.02**

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV 020	459.00	147.00	3.122	0.30	
JLV 020	416.00	137.00	3.036	0.29	
JLV 020	408.00	129.00	3.163	0.31	
No.of Samples	3	**Mean**	3.11	**0.30**	**0.02**

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV 021	242.00	81.00	2.988	0.29	
JLV 021	231.00	75.00	3.080	0.30	
JLV 021	211.00	74.00	2.851	0.27	
No.of Samples	3	**Mean**	2.97	**0.29**	**0.02**

Expected dialysate T of 2.5 ng/mL

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV 022	61.00	155.00	0.394	0.03	
JLV 022	63.00	161.00	0.391	0.03	
JLV 022	58.00	150.00	0.387	0.03	
No.of Samples	3	**Mean**	0.39	**0.03**	**0.03**
Sample Name	Drug Response	Std. Response	Response Ratio	**Drug Conc.**	**Std.Err.**
JLV 023	3561.00	188.00	18.941	1.89	
JLV 023	3666.00	188.00	19.500	1.95	
JLV 023	3337.00	177.00	18.853	1.88	
No.of Samples	3	**Mean**	19.10	**1.91**	**0.06**
Sample Name	Drug Response	Std. Response	Response Ratio	**Drug Conc.**	**Std.Err.**
JLV 024	3259.00	166.00	19.633	1.96	
JLV 024	3007.00	164.00	18.335	1.83	
JLV 024	2822.00	150.00	18.813	1.88	
No.of Samples	3	**Mean**	18.93	**1.89**	**0.06**
Sample Name	Drug Response	Std. Response	Response Ratio	**Drug Conc.**	**Std.Err.**
JLV 025	3856.00	193.00	19.979	1.99	
JLV 025	3656.00	185.00	19.762	1.97	
JLV 025	3450.00	185.00	18.649	1.86	
No.of Samples	3	**Mean**	19.46	**1.94**	**0.07**
Sample Name	Drug Response	Std. Response	Response Ratio	**Drug Conc.**	**Std.Err.**
JLV 026	4218.00	223.00	18.915	1.89	
JLV 026	4329.00	227.00	19.070	1.90	
JLV 026	4068.00	211.00	19.280	1.92	
No.of Samples	3	**Mean**	19.09	**1.90**	**0.06**
Sample Name	Drug Response	Std. Response	Response Ratio	**Drug Conc.**	**Std.Err.**
JLV 027	2392.00	114.00	20.982	2.09	
JLV 027	2408.00	129.00	18.667	1.86	
JLV 027	2162.00	110.00	19.655	1.96	
No.of Samples	3	**Mean**	19.77	**1.97**	**0.07**

8.4 Testosterone aqueous solution with alcohol at room temperature

Appendix 8.4 Testosterone aqueous solution with alcohol at room temperature

Compound : Testosterone **Conc. units: ng/mL**

Drug Conc.	Drug Response	Internal Std. Response	Response Ratio	Percentage Error
0.00	15.00	4501.00	0.003	
1.00	1519.00	3654.00	0.416	-4.20
2.00	4386.00	5070.00	0.865	0.30
5.00	9557.00	4383.00	2.180	1.47
10.00	21691.00	5070.00	4.278	-0.34

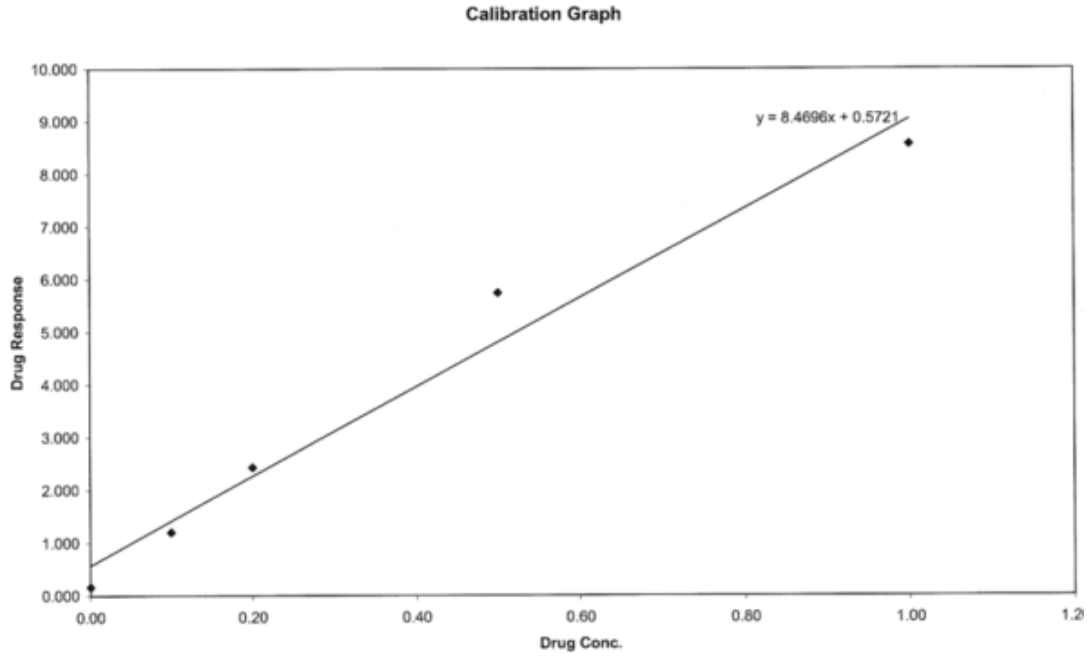
REGRESSION STATISTICS

Correlation Coeff.	**0.9999**	**Percentage Fit**	**99.99**
Adjusted R Square	0.9998	Std. Deviation	0.0227
xmean	3.6000	No. Observations	5
ymean	1.5486	X-Intercept	-0.0116

	Deg.of Freedom	Sum of Squares	Mean Squares	F Ratio
Regression	1	11.9875	11.9875	23314
Residual	3	0.0015	0.0005	
Total	4	11.9890	2.9973	

	Coefficients	Std. Err. Estimates
Y-Intercept	0.0050	0.0143
Slope	0.4288	0.0028

Sample Name	Drug Response	Std. Response	Response Ratio	Drug Conc.	Std.Err.
JLV074	19897.00	10173.00	1.956	4.55	
JLV074	19263.00	9988.00	1.929	4.49	
JLV074	23052.00	11873.00	1.942	4.52	
No.of Samples	3	**Mean**	1.94	**4.52**	**0.04**


Calibration Graph
y = 8.4696x + 0.5721
Drug Response
Drug Conc.
10.000
9.000
8.000
7.000
6.000
5.000
4.000
3.000
2.000
1.000
0.000
0.00
0.20
0.40
0.60
0.80
1.00

8.5 Testosterone & SHBG aqueous solution at room temperature

Appendix 8.5 Testosterone & SHBG aqueous solution at room temperature

Compound : Testosterone **Conc. units: ng/mL**

Drug Conc.	Drug Response	Internal Std. Response	Response Ratio	Percentage Error
0.00	10.00	74.00	0.135	
0.10	163.00	161.00	1.012	-19.77
0.20	415.00	170.00	2.441	21.94
0.50	714.00	151.00	4.728	1.18
1.00	1451.00	162.00	8.957	-0.98

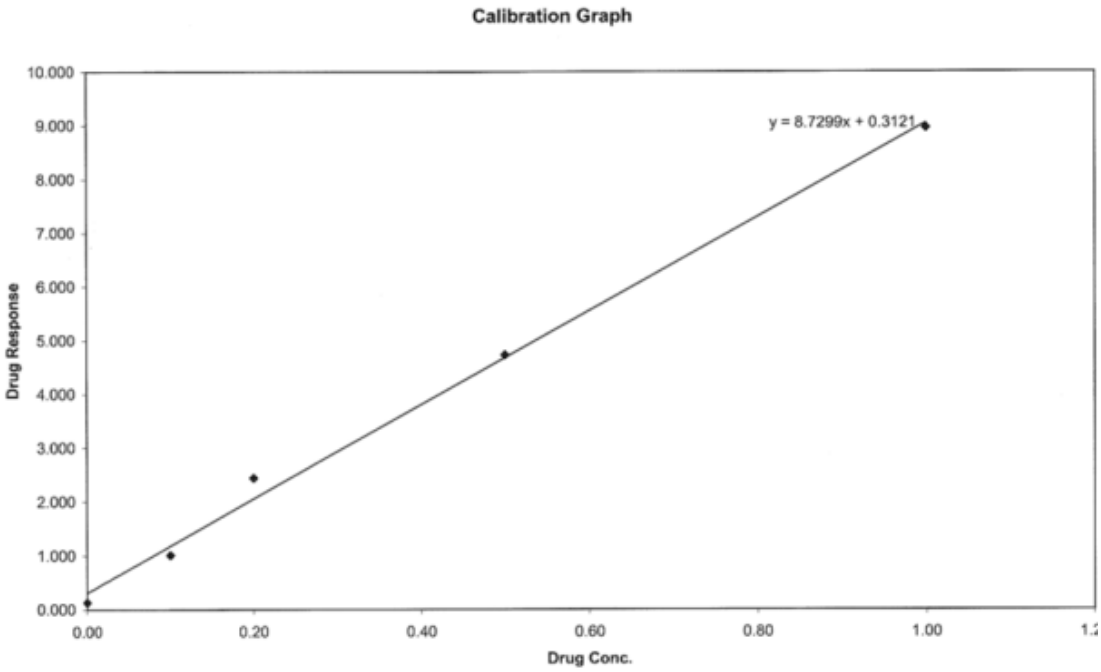
REGRESSION STATISTICS

Correlation Coeff.	**0.9978**	**Percentage Fit**	**99.56**
Adjusted R Square	0.9942	Std. Deviation	0.2694
xmean	0.3600	No. Observations	5
ymean	3.4548	X-Intercept	-0.0357

	Deg.of Freedom	Sum of Squares	Mean Squares	F Ratio
Regression	1	49.6892	49.6892	684
Residual	3	0.2178	0.0726	
Total	4	49.9070	12.4767	

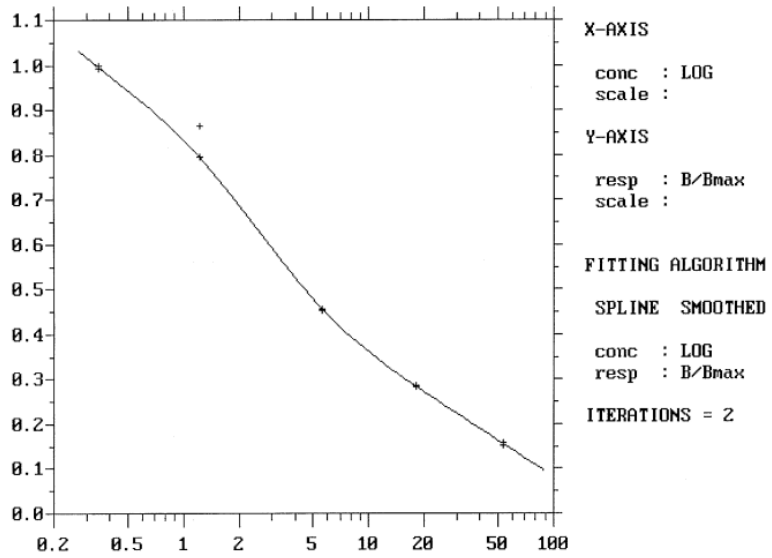
	Coefficients	Std. Err. Estimates
Y-Intercept	0.3121	0.1701
Slope	8.7299	0.3337

Sample Name	Drug Response	Std. Response	Response Ratio	**Drug Conc.**	**Std.Err.**
JLV076	594.00	987.00	0.602	0.03	
JLV076	460.00	829.00	0.555	0.03	
JLV076	454.00	758.00	0.599	0.03	
No.of Samples	3	**Mean**	0.59	**0.03**	**0.03**


Calibration Graph
y = 8.7299x + 0.3121
Drug Response
Drug Conc.
10.000
9.000
8.000
7.000
6.000
5.000
4.000
3.000
2.000
1.000
0.000
0.00
0.20
0.40
0.60
0.80
1.00

8.6 Serum testosterone equilibrium at room temperature

Appendix 8.6 Serum testosterone equilibrium at room temperature

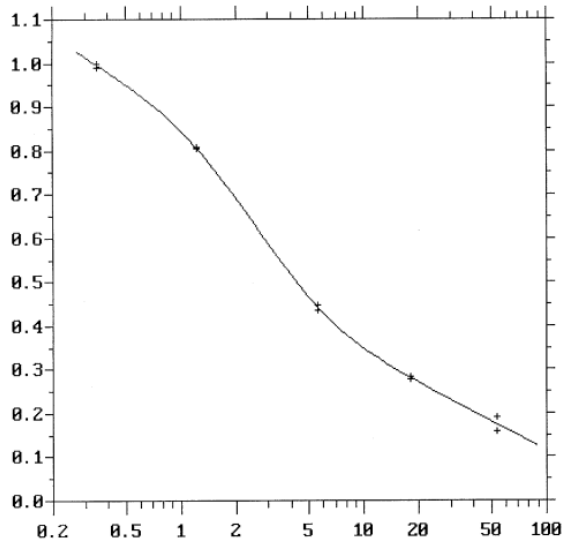


Standards:

SEQ	CODE	COUNTS	CONC	UNIT	%CV
1	BLANK	76386			
2	BLANK	76123			
AVG.	**BLANK**	**76254**		**nmol/L**	**0.2**
3	STD	61600	0.350		
4	STD	61110	0.350		
AVG.	**STD**	**61355**	**0.350**	**nmol/L**	**0.6**
5	STD	53196	1.230		
6	STD	49106	1.230		
AVG.	**STD**	**51151**	**1.230**	**nmol/L**	**5.7**
7	STD	27982	5.600		
8	STD	28102	5.600		
AVG.	**STD**	**28042**	**5.600**	**nmol/L**	**0.3**
9	STD	17375	18.000		
10	STD	17655	18.000		
AVG.	**STD**	**17515**	**18.000**	**nmol/L**	**1.1**
11	STD	9423	53.300		
12	STD	9740	53.300		
AVG.	**STD**	**9582**	**53.300**	**nmol/L**	**2.3**

Samples:

17	3	3	16688	20.098			.....	JLV101
18	3	3	16624	20.280			.....	JLV101
AVG.	**3**	**3**	**16656**	**20.189**	**nmol/L**	**0.6**	.....	**JLV101**



X-AXIS

conc : LOG
scale :

Y-AXIS

resp : B/Bmax
scale :

FITTING ALGORITHM

SPLINE SMOOTHED

conc : LOG
resp : B/Bmax

ITERATIONS = 1

Standards:

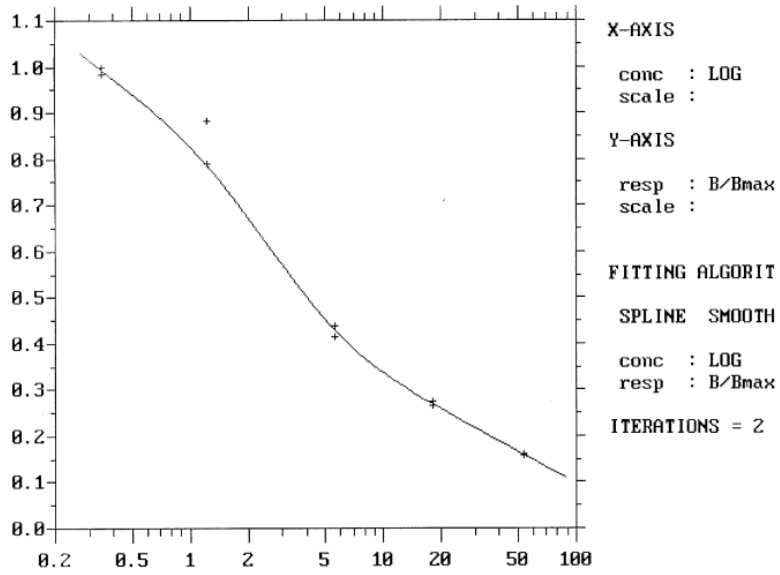
SEQ	CODE	COUNTS	CONC	UNIT	%CV
1	BLANK	69291			
2	BLANK	72487			
AVG.	**BLANK**	**70889**		**nmol/L**	**3.2**
3	STD	60790	0.350		
4	STD	61344	0.350		
AVG.	**STD**	**61067**	**0.350**	**nmol/L**	**0.6**
5	STD	49305	1.230		
6	STD	49487	1.230		
AVG.	**STD**	**49396**	**1.230**	**nmol/L**	**0.3**
7	STD	26684	5.600		
8	STD	27443	5.600		
AVG.	**STD**	**27064**	**5.600**	**nmol/L**	**2.0**
9	STD	17086	18.000		
10	STD	17342	18.000		
AVG.	**STD**	**17214**	**18.000**	**nmol/L**	**1.1**
11	STD	11788	53.300		
12	STD	9615	53.300		
AVG.	**STD**	**10702**	**53.300**	**nmol/L**	**14.4**

Samples:

17	3	3	20019	11.838			.....	JLV113
18	3	3	20099	11.711			.....	JLV113
AVG.	**3**	**3**	**20059**	**11.774**	**nmol/L**	**0.8**	.....	**JLV113**

8.7 Serum testosterone with 350 mg% alcohol at room temperature

Appendix 8.7 Serum testosterone with 350 mg% alcohol at room temperature



Standards:

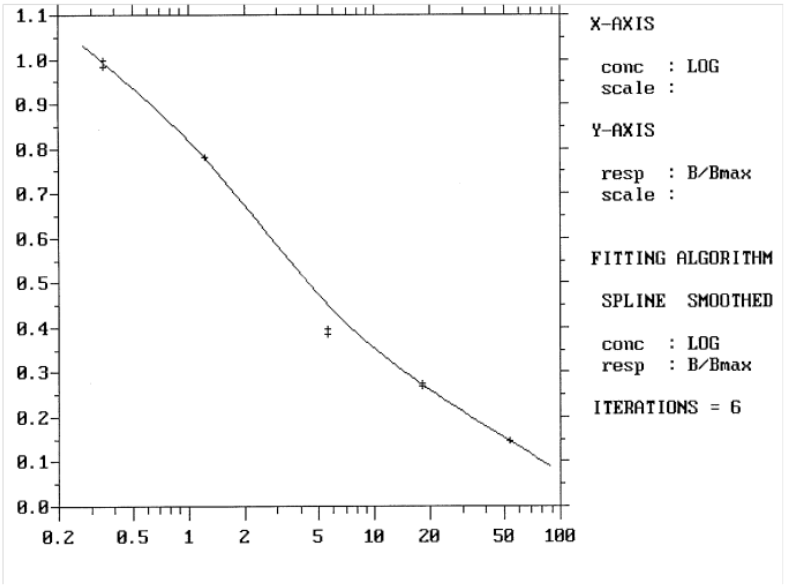
SEQ	CODE	COUNTS	CONC	UNIT	%CV
1	BLANK	75140			
2	BLANK	75228			
AVG.	BLANK	75184		nmol/L	0.1
3	STD	64387	0.350		
4	STD	63339	0.350		
AVG.	STD	63863	0.350	nmol/L	1.2
5	STD	56744	1.230		
6	STD	50774	1.230		
AVG.	STD	53759	1.230	nmol/L	7.9
7	STD	28149	5.600		
8	STD	26657	5.600		
AVG.	STD	27403	5.600	nmol/L	3.9
9	STD	17603	18.000		
10	STD	17005	18.000		
AVG.	STD	17304	18.000	nmol/L	2.4
11	STD	10442	53.300		
12	STD	10078	53.300		
AVG.	STD	10260	53.300	nmol/L	2.5

Samples:

SEQ	PAT	CODE	COUNTS	CONC	UNIT	%CV		LINE	REMARK
17	3	3	16334	20.770				.....	JLV090
18	3	3	17033	18.710				.....	JLV090
AVG.	3	3	16684	19.740	nmol/L	7.4		.....	JLV090
19	4	4	3782	148.283			> STD	.....	JLV091
20	4	4	21855	9.774				.....	JLV091
AVG.	4	4	12818	79.029	nmol/L	123.9	%CV !	.....	JLV091
21	5	5	25358	6.798				.....	JLV096
22	5	5	26560	6.112				.....	JLV096
AVG.	5	5	25959	6.455	nmol/L	7.5		.....	JLV096

8.8 Serum testosterone with 350 mg% alcohol at 37ºC

Appendix 8.8 Serum testosterone with 350 mg% alcohol at 37ºC

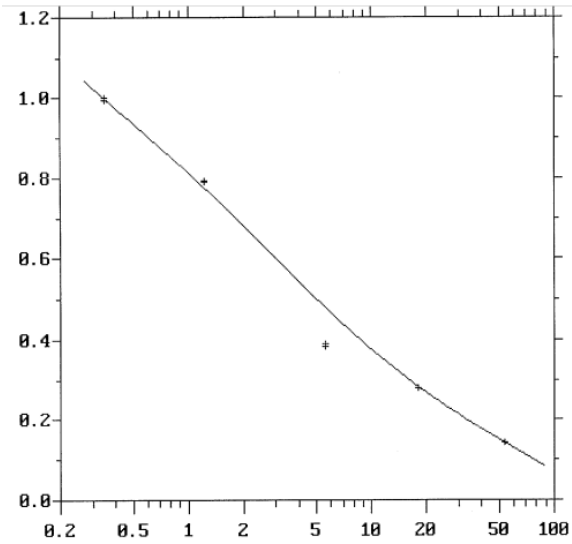


Standards:

SEQ	CODE	COUNTS	CONC	UNIT	%CV
1	BLANK	75901			
2	BLANK	73668			
AVG.	**BLANK**	**74784**		nmol/L	2.1
3	STD	60443	0.350		
4	STD	61523	0.350		
AVG.	**STD**	**60983**	**0.350**	nmol/L	1.3
5	STD	48050	1.230		
6	STD	48140	1.230		
AVG.	**STD**	**48095**	**1.230**	nmol/L	0.1
7	STD	24482	5.600		
8	STD	23632	5.600		
AVG.	**STD**	**24057**	**5.600**	nmol/L	2.5
9	STD	16927	18.000		
10	STD	16529	18.000		
AVG.	**STD**	**16728**	**18.000**	nmol/L	1.7
11	STD	8925	53.300		
12	STD	8999	53.300		
AVG.	**STD**	**8962**	**53.300**	nmol/L	0.6

Samples:

SEQ	PAT	CODE	COUNTS	CONC	UNIT	%CV	LINE	REMARK
15	2	2	15885	20.056			.....	JLV116
16	2	2	16185	19.274			.....	JLV116
AVG.	**2**	**2**	**16035**	**19.665**	nmol/L	2.8	.....	JLV116
39	14	14	32525	3.838			.....	JLV129
40	14	14	32563	3.827			.....	JLV129
AVG.	**14**	**14**	**32544**	**3.832**	nmol/L	0.2	.....	JLV129



Standards:

SEQ	CODE	COUNTS	CONC	UNIT	%CV
1	BLANK	76866			
2	BLANK	76073			
AVG.	BLANK	76470		nmol/L	0.7
3	STD	61382	0.350		
4	STD	61723	0.350		
AVG.	STD	61552	0.350	nmol/L	0.4
5	STD	48706	1.230		
6	STD	49044	1.230		
AVG.	STD	48875	1.230	nmol/L	0.5
7	STD	24162	5.600		
8	STD	23728	5.600		
AVG.	STD	23945	5.600	nmol/L	1.3
9	STD	17211	18.000		
10	STD	17343	18.000		
AVG.	STD	17277	18.000	nmol/L	0.5
11	STD	8896	53.300		
12	STD	8858	53.300		
AVG.	STD	8877	53.300	nmol/L	0.3

Samples:

SEQ	PAT	CODE	COUNTS	CONC	UNIT	%CV	LINE	REMARK
37	13	13	34612	3.678			.....	JLV123
38	13	13	34323	3.766			.....	JLV123
AVG.	13	13	34468	3.722	nmol/L	1.7	.....	JLV123

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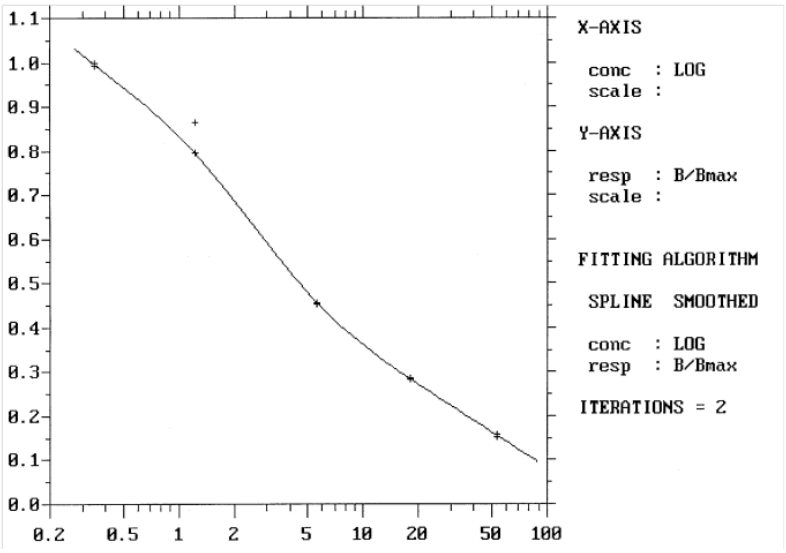
8.9 Serum testosterone with 700 mg% alcohol at room temperature

Appendix 8.9 Serum testosterone with 700 mg% alcohol at room temperature



Standards:

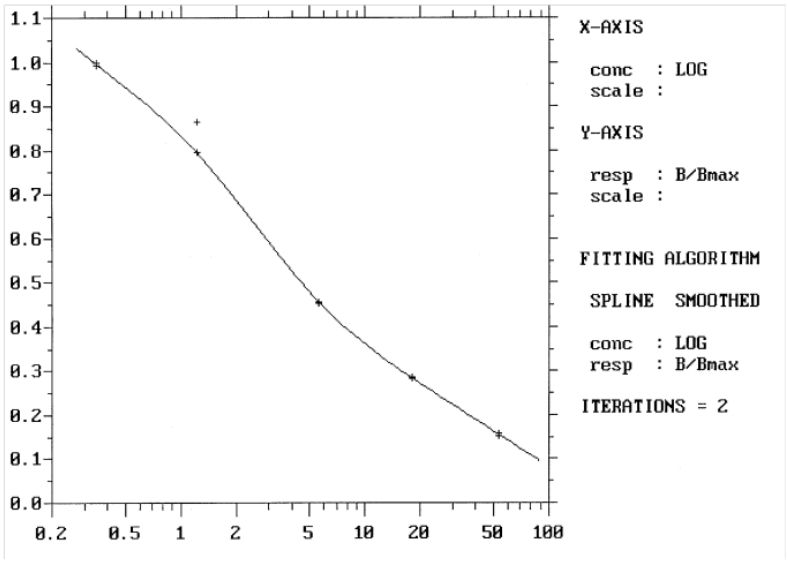
SEQ	CODE	COUNTS	CONC	UNIT	%CV
1	BLANK	76386			
2	BLANK	76123			
AVG.	BLANK	76254		nmol/L	0.2
3	STD	61600	0.350		
4	STD	61110	0.350		
AVG.	STD	61355	0.350	nmol/L	0.6
5	STD	53196	1.230		
6	STD	49106	1.230		
AVG.	STD	51151	1.230	nmol/L	5.7
7	STD	27982	5.600		
8	STD	28102	5.600		
AVG.	STD	28042	5.600	nmol/L	0.3
9	STD	17375	18.000		
10	STD	17655	18.000		
AVG.	STD	17515	18.000	nmol/L	1.1
11	STD	9423	53.300		
12	STD	9740	53.300		
AVG.	STD	9582	53.300	nmol/L	2.3

Samples:

SEQ	PAT	CODE	COUNTS	CONC	UNIT	%CV	LINE	REMARK
19	4	4	25312	7.193			.....	JLV107
20	4	4	25329	7.180			.....	JLV107
AVG.	4	4	25320	7.186	nmol/L	0.1	.....	JLV107

8.10 Serum testosterone in a 250 mL volume of buffer with 350 mg% of EtOH at room temperature

Appendix 8.10 Serum testosterone in a 250 mL volume of buffer with 350 mg% of EtOH at room temperature



Standards:

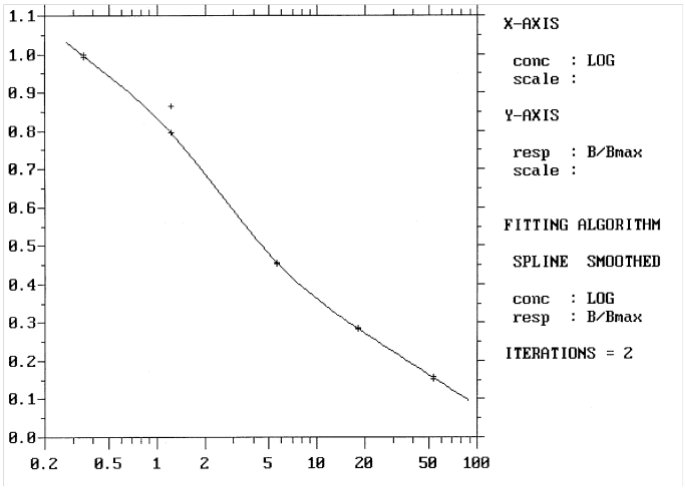
SEQ	CODE	COUNTS	CONC	UNIT	%CV
1	BLANK	76386			
2	BLANK	76123			
AVG.	BLANK	76254		nmol/L	0.2
3	STD	61600	0.350		
4	STD	61110	0.350		
AVG.	STD	61355	0.350	nmol/L	0.6
5	STD	53196	1.230		
6	STD	49106	1.230		
AVG.	STD	51151	1.230	nmol/L	5.7
7	STD	27982	5.600		
8	STD	28102	5.600		
AVG.	STD	28042	5.600	nmol/L	0.3
9	STD	17375	18.000		
10	STD	17655	18.000		
AVG.	STD	17515	18.000	nmol/L	1.1
11	STD	9423	53.300		
12	STD	9740	53.300		
AVG.	STD	9582	53.300	nmol/L	2.3

Samples:

SEQ	PAT	CODE	COUNTS	CONC	UNIT	%CV	LINE	REMARK
21	5	5	26141	6.645			.....	JLV105
22	5	5	26444	6.462			.....	JLV105
AVG.	5	5	26292	6.553	nmol/L	2.0	.....	JLV105

8.11 Serum testosterone in tube dialysis at room temperature

Appendix 8.11 Serum testosterone in tube dialysis at room temperature



Standards:

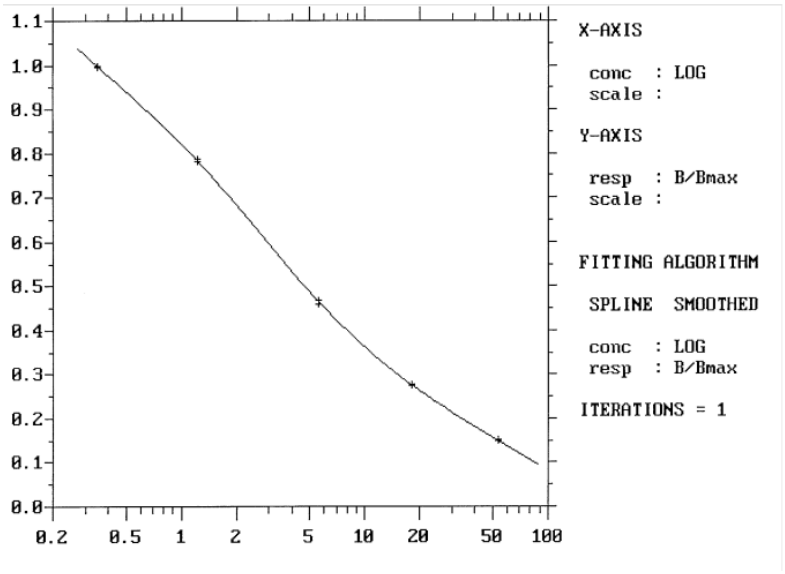
SEQ	CODE	COUNTS	CONC	UNIT	%CV
1	BLANK	76386			
2	BLANK	76123			
AVG.	BLANK	76254		nmol/L	0.2
3	STD	61600	0.350		
4	STD	61110	0.350		
AVG.	STD	61355	0.350	nmol/L	0.6
5	STD	53196	1.230		
6	STD	49106	1.230		
AVG.	STD	51151	1.230	nmol/L	5.7
7	STD	27982	5.600		
8	STD	28102	5.600		
AVG.	STD	28042	5.600	nmol/L	0.3
9	STD	17375	18.000		
10	STD	17655	18.000		
AVG.	STD	17515	18.000	nmol/L	1.1
11	STD	9423	53.300		
12	STD	9740	53.300		
AVG.	STD	9582	53.300	nmol/L	2.3

Samples:

SEQ	PAT	CODE	COUNTS	CONC	UNIT	%CV	LINE	REMARK
23	6	6	20848	11.724			.....	JLV106
24	6	6	20148	12.785			.....	JLV106
AVG.	6	6	20498	12.255	nmol/L	6.1	.....	JLV106

8.12 Serum testosterone in tube dialysis at 37ºC

Appendix 8.12 Serum testosterone in tube dialysis at 37ºC



Standards:

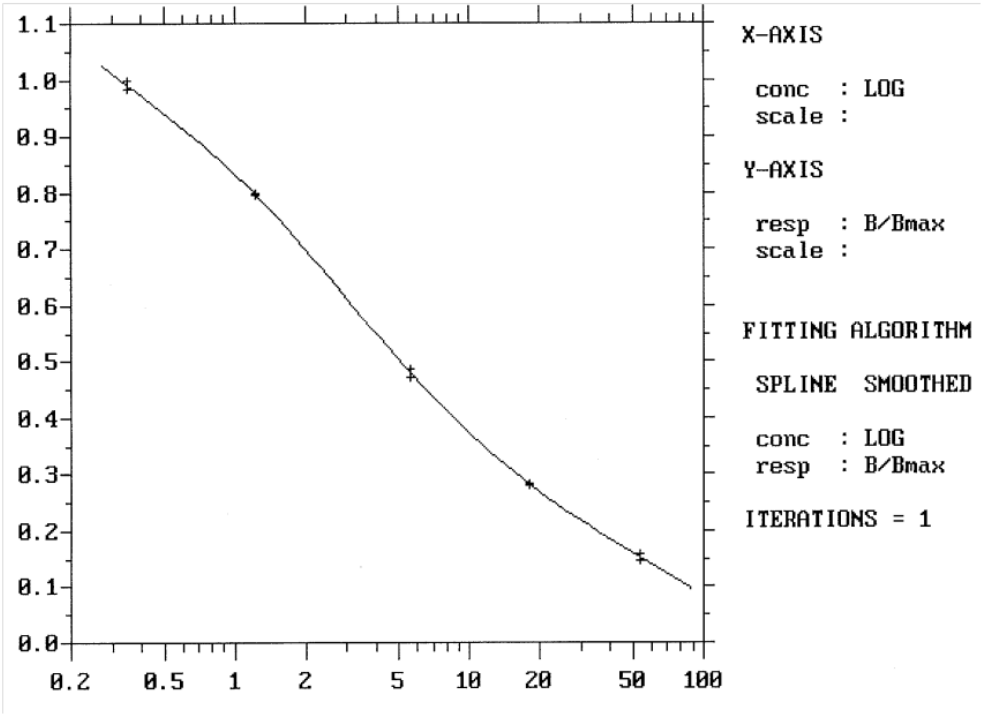
SEQ	CODE	COUNTS	CONC	UNIT	%CV
1	BLANK	65482			
2	BLANK	65238			
AVG.	**BLANK**	**65360**		nmol/L	0.3
3	STD	55680	0.350		
4	STD	55902	0.350		
AVG.	**STD**	**55791**	**0.350**	nmol/L	0.3
5	STD	43582	1.230		
6	STD	43947	1.230		
AVG.	**STD**	**43764**	**1.230**	nmol/L	0.6
7	STD	26224	5.600		
8	STD	25696	5.600		
AVG.	**STD**	**25960**	**5.600**	nmol/L	1.4
9	STD	15477	18.000		
10	STD	15318	18.000		
AVG.	**STD**	**15398**	**18.000**	nmol/L	0.7
11	STD	8310	53.300		
12	STD	8443	53.300		
AVG.	**STD**	**8376**	**53.300**	nmol/L	1.1

Samples:

SEQ	PAT	CODE	COUNTS	CONC	UNIT	%CV	LINE	REMARK
13	1	1	14788	19.572			.....	JLV118
14	1	1	14415	20.616			.....	JLV118
AVG.	**1**	**1**	**14602**	**20.094**	nmol/L	3.7	.....	**JLV118**
15	2	2	29261	4.180			.....	JLV157
16	2	2	29475	4.105			.....	JLV157
AVG.	**2**	**2**	**29368**	**4.143**	nmol/L	1.3	.....	**JLV157**
17	3	3	30664	3.711			.....	JLV158
18	3	3	28900	4.312			.....	JLV158
AVG.	**3**	**3**	**29782**	**4.012**	nmol/L	10.6 %CV !	.....	**JLV158**

8.13 Ultrafiltration results

Appendix 8.13 Ultrafiltration results



Standards:

SEQ	CODE	COUNTS	CONC	UNIT	%CV
1	BLANK	63882			
2	BLANK	63418			
AVG.	BLANK	63650		nmol/L	0.5
3	STD	54576	0.350		
4	STD	53622	0.350		
AVG.	STD	54099	0.350	nmol/L	1.2
5	STD	43569	1.230		
6	STD	43414	1.230		
AVG.	STD	43492	1.230	nmol/L	0.3
7	STD	26568	5.600		
8	STD	25725	5.600		
AVG.	STD	26146	5.600	nmol/L	2.3
9	STD	15371	18.000		
10	STD	15420	18.000		
AVG.	STD	15396	18.000	nmol/L	0.2
11	STD	8003	53.300		
12	STD	8608	53.300		
AVG.	STD	8306	53.300	nmol/L	5.2

Samples:

SEQ	PAT	CODE	COUNTS	CONC	UNIT	%CV		LINE	REMARK
13	1	1	20079	10.257				.....	JLV121
14	1	1	20050	10.289				.....	JLV121
AVG.	**1**	**1**	**20064**	**10.273**	**nmol/L**	**0.2**		**.....**	**JLV121**
17	3	3	20691	9.598				.....	JLV149
18	3	3	20428	9.875				.....	JLV149
AVG.	**3**	**3**	**20560**	**9.737**	**nmol/L**	**2.0**		**.....**	**JLV149**
19	4	4	68610	0.052			< STD	.....	JLV150
20	4	4	69986	0.043			< STD	.....	JLV150
AVG.	**4**	**4**	**69298**	**0.047**	**nmol/L**	**12.8**	**%CV !**	**.....**	**JLV150**
23	6	6	19159	11.362				.....	JLV151
24	6	6	19866	10.499				.....	JLV151
AVG.	**6**	**6**	**19512**	**10.931**	**nmol/L**	**5.6**		**.....**	**JLV151**
25	7	7	70923	0.038			< STD	.....	JLV152
26	7	7	70896	0.038			< STD	.....	JLV152
AVG.	**7**	**7**	**70910**	**0.038**	**nmol/L**	**0.3**	**< STD**	**.....**	**JLV152**
29	9	9	19749	10.635				.....	JLV153
30	9	9	19947	10.407				.....	JLV153
AVG.	**9**	**9**	**19848**	**10.521**	**nmol/L**	**1.5**		**.....**	**JLV153**
31	10	10	68639	0.051			< STD	.....	JLV154
32	10	10	68824	0.050			< STD	.....	JLV154
AVG.	**10**	**10**	**68732**	**0.051**	**nmol/L**	**1.7**	**< STD**	**.....**	**JLV154**
35	12	12	20476	9.825				.....	JLV155
36	12	12	19843	10.526				.....	JLV155
AVG.	**12**	**12**	**20160**	**10.176**	**nmol/L**	**4.9**		**.....**	**JLV155**
37	13	13	72300	0.032			< STD	.....	JLV156
38	13	13	72266	0.032			< STD	.....	JLV156
AVG.	**13**	**13**	**72283**	**0.032**	**nmol/L**	**0.3**	**< STD**	**.....**	**JLV156**

8.14 Concentration of internal standard

Appendix 8.14 Concentration of internal standard

Deuterated steroid	Amount in ampoule (mg)	Final concentration (μg/mL)
d_3-testosterone	0.902	90.2
d_3-epitestosterone	0.925	92.5
$d_3$5α-DHT	0.83	83

8.15 Internal standard mixture (stock solution)

Appendix 8.15 Internal standard mixture (stock solution)

Deuterated steroid	Amount to be added (μg)	Final concentration (μg/mL)	Addition made
d_3-testosterone	100	0.5	1.11 mL o f 90.2 μg/mL
d_3-epitestosterone	100	0.5	1.10 mL o f 92.5 μg/mL
$d_3$5α-DHT	64	0.32	0.770 mL of 83 μg/mL

8.16 Urinary T/E, T, E and 5α-DHT concentrations (ng/mL) for females after 4 units

Appendix 8.16 Urinary T/E, T, E and 5α-DHT concentrations (ng/mL) for females after 4 units

Sample	H after intake	T/E	T conc	E conc	5α-DHT conc	SG
T1F	0	0.30	9.71	35.8	9.48	1.012
T1F	1	0.67	11.8	14.9	5.00	1.002
T1F	2	0.75	10.8	12.8	2.50	1.004
T1F	3	0.63	6.42	9.74	3.56	1.008
T1F	4	0.52	8.97	14.1	2.50	1.004
T1F	5	0.28	6.89	22.1	3.84	1.015
T1F	6	0.26	7.08	24.4	4.73	1.021
T1F	7	0.29	12.0	38.6	10.1	1.022
T1F	10	0.32	6.82	19.4	5.07	1.007
T1F	24	0.27	6.83	23.8	6.03	1.014
T1F	48	0.48	13.0	25.7	9.62	1.003
T2F	0	1.20	13.5	12.1	3.65	1.006
T2F	1	2.04	15.4	5.00	5.00	1.002
T2F	2	2.94	14.9	5.96	2.00	1.005
T2F	3	3.50	24.3	6.15	3.80	1.009
T2F	4	3.43	30.3	6.92	5.29	1.008
T2F	5	2.10	13.2	5.12	3.74	1.012

T2F	6	1.47	11.0	4.88	3.73	1.014
T2F	7	1.54	12.3	13.5	3.63	1.007
T2F	10	1.88	27.2	9.26	5.23	1.005
T2F	24	1.06	7.51	8.22	3.52	1.007
T2F	48	0.80	6.02	6.55	3.15	1.007
T3F	0	0.66	11.1	16.8	6.81	1.004
T3F	1	2.44	36.8	16.4	3.33	1.003
T3F	2	2.41	14.6	6.67	2.00	1.005
T3F	3	2.68	72.8	29.9	10.0	1.001
T3F	4	2.95	81.8	29.9	10.0	1.001
T3F	5	3.13	33.2	11.7	4.42	1.005
T3F	6	2.25	25.0	10.5	3.50	1.011
T3F	7	1.61	19.7	13.5	4.97	1.013
T3F	10	1.03	16.7	17.8	5.62	1.010
T3F	24	0.66	11.3	16.8	5.70	1.010
T3F	48	0.58	26.9	53.3	20.3	1.003
T4F	0	0.43	5.18	11.6	3.90	1.007
T4F	1	1.12	8.33	7.98	2.00	1.005
T4F	2	1.19	9.87	8.71	2.18	1.014
T4F	3	1.63	17.5	10.1	2.38	1.013
T4F	4	1.79	20.3	11.3	2.65	1.01
T4F	5	1.48	20.0	12.6	3.69	1.015
T4F	6	0.86	11.1	12.3	3.37	1.018
T4F	7	0.63	12.5	19.4	4.55	1.020
T4F	10	0.44	6.47	13.9	2.32	1.020
T4F	24	0.50	7.64	14.5	2.73	1.009
T4F	48	0.45	5.62	11.9	1.25	1.008
T5F	0	0.46	29.1	66.1	6.64	1.011
T5F	1	1.28	40.9	30.0	5.00	1.002
T5F	2	1.68	90.1	54.6	10.0	1.001
T5F	3	1.68	67.3	34.3	6.84	1.004
T5F	4	1.52	55.8	31.6	6.74	1.005
T5F	5	1.02	34.7	34.4	6.51	1.007
T5F	6	0.68	31.1	50.4	5.85	1.012
T5F	7	0.58	31.8	48.9	8.42	1.009
T5F	10	0.58	30.3	51.5	10.5	1.002
T5F	24	0.56	16.9	30.3	3.33	1.003
T5F	48	0.58	20.2	32.4	4.86	1.008
T6F	0	1.14	19.5	16.5	9.88	1.003
T6F	1	1.94	16.5	9.10	7.19	1.003
T6F	2	2.40	23.4	7.51	7.84	1.013
T6F	3	2.48	27.2	8.58	8.17	1.015
T6F	4	1.55	37.6	14.3	12.8	1.023
T6F	5	0.86	17.3	11.0	11.6	1.022
T6F	6	0.83	15.0	9.03	8.19	1.021
T6F	7	1.18	20.9	11.5	11.1	1.017
T6F	10	1.42	11.0	6.65	7.39	1.008

T6F	24	1.29	14.9	9.78	8.73	1.007
T6F	48	1.30	11.3	8.33	10.1	1.006
T7F	0	0.54	9.53	16.0	2.47	1.012
T7F	1	1.38	26.9	19.2	5.00	1.002
T7F	2	1.18	20.4	16.0	2.60	1.008
T7F	3	1.11	34.1	20.2	2.68	1.018
T7F	4	1.12	20.0	14.5	2.56	1.013
T7F	5	0.73	9.04	11.7	1.67	1.006
T7F	6	0.49	8.31	14.4	2.14	1.011
T7F	7	0.39	11.0	20.0	2.45	1.018
T7F	10	0.45	8.73	15.2	2.77	1.011
T7F	24	0.35	14.3	29.6	10.3	1.019
T7F	48	3.06	127	33.4	4.05	1.013
T8F	0	0.35	3.33	14.2	7.93	1.003
T8F	1	0.88	12.0	11.6	5.00	1.002
T8F	2	1.14	15.0	12.4	2.00	1.005
T8F	3	1.31	27.0	17.5	4.28	1.014
T8F	4	1.07	16.1	13.3	3.00	1.01
T8F	5	0.64	11.3	15.9	12.5	1.008
T8F	6	0.59	10.1	15.7	3.31	1.010
T8F	7	0.51	14.0	21.4	5.81	1.011
T8F	10	0.55	12.4	20.3	6.24	1.006
T8F	24	0.34	7.99	19.0	4.16	1.009
T8F	48	0.42	10.1	22.4	6.30	1.004
T9F	0	0.89	11.0	11.0	5.45	1.023
T9F	1	2.58	25.7	9.05	3.33	1.003
T9F	2	3.49	51.5	11.8	6.78	1.026
T9F	3	3.42	59.8	12.8	6.66	1.031
T9F	4	3.16	63.6	15.1	9.29	1.03
T9F	5	1.74	32.6	14.8	7.27	1.027
T9F	6	1.36	19.0	10.6	5.45	1.016
T9F	7	1.27	12.4	10.9	3.33	1.003
T9F	10	1.17	25.0	17.2	8.52	1.026
T9F	24	0.94	6.85	6.47	2.18	1.017
T9F	48	0.65	11.6	14.6	6.17	1.019
T10F	0	1.19	26.5	16.3	10.4	1.026
T10F	1	2.46	13.5	4.43	4.65	1.014
T10F	2	2.66	12.2	4.31	3.10	1.009
T10F	3	2.26	17.7	6.53	4.90	1.012
T10F	4	1.70	8.74	13.5	8.20	1.009
T10F	5	1.62	10.3	6.54	6.78	1.005
T10F	6	1.62	13.8	7.73	7.38	1.007
T10F	7	1.64	14.2	8.16	10.7	1.008
T10F	10	1.69	19.7	8.48	14.7	1.021
T10F	24	0.99	24.3	16.7	15.9	1.027
T10F	48	1.24	11.3	7.74	7.50	1.013

8.17 Urinary T/E, T, E and 5α-DHT concentrations (ng/mL) for females after 8 units

Appendix 8.17 Urinary T/E, T, E and 5α-DHT concentrations (ng/mL) for females after 8 units

Sample	H after intake	T/E	T conc	E conc	5α-DHT conc	SG
T1F	0	0.60	7.02	12.1	11.68	1.019
T1F	1	1.22	5.00	5.00	5.00	1.002
T1F	2	1.33	7.95	3.33	3.33	1.003
T1F	3	1.89	9.71	4.86	3.75	1.007
T1F	4	2.25	14.53	6.22	5.20	1.012
T1F	5	2.82	17.13	2.75	5.49	1.01
T1F	6	1.82	13.23	7.97	6.35	1.011
T1F	7	1.15	10.08	9.56	7.35	1.009
T1F	10	0.87	11.26	12.8	8.11	1.014
T1F	24	0.59	4.20	8.48	6.86	1.018
T1F	48	0.62	3.33	8.09	3.3	1.003
T2F	0	1.09	20.7	19.5	1.25	1.008
T2F	1	1.29	7.95	3.33	3.33	1.003
T2F	2	1.62	12.0	3.33	3.33	1.003
T2F	3	3.11	24.6	9.32	2.50	1.004
T2F	4	3.44	28.4	8.72	2.66	1.008
T2F	5	4.62	34.9	8.62	3.08	1.013
T2F	6	4.21	35.3	8.11	5.17	1.007
T2F	7	4.67	52.0	13.4	5.30	1.012
T2F	10	2.01	26.7	13.5	9.58	1.003
T2F	24	0.77	9.39	10.9	2.50	1.004
T2F	48	0.09	15.4	14.6	2.00	1.005
T3F	0	0.61	17.2	24.9	7.58	1.006
T3F	1	1.48	18.9	10.9	3.33	1.003
T3F	2	2.08	27.5	12.4	5.00	1.002
T3F	3	2.45	36.0	13.2	5.00	1.002
T3F	4	2.68	37.4	12.7	5.00	1.002
T3F	5	2.39	30.4	12.3	3.33	1.003
T3F	6	2.54	38.2	14.9	4.57	1.005
T3F	7	1.78	31.0	16.4	3.33	1.003
T3F	10	0.98	12.3	11.1	3.33	1.003
T3F	24	0.55	11.2	17.7	5.28	1.009
T3F	48	0.47	7.89	14.7	4.78	1.009
T4F	0	0.91	5.59	5.62	1.99	1.015
T4F	1	1.50	3.33	3.33	3.33	1.003
T4F	2	1.45	5.00	5.00	5.00	1.002
T4F	3	2.07	8.24	3.54	1.43	1.007
T4F	4	2.60	8.07	2.81	1.11	1.009
T4F	5	3.26	9.94	2.95	1.84	1.014
T4F	6	3.67	18.6	5.71	2.46	1.017

T4F	7	2.74	16.4	5.19	2.04	1.014
T4F	10	0.89	5.00	6.02	5.00	1.002
T4F	24	0.87	8.43	8.38	0.5	1.023
T4F	48	0.88	5.44	5.37	1.67	1.006
T5F	0	0.72	8.77	10.3	2.00	1.005
T5F	1	2.33	17.6	3.33	3.33	1.003
T5F	2	2.91	20.9	5.00	5.00	1.002
T5F	3	4.39	21.7	5.00	5.00	1.002
T5F	4	5.21	18.3	3.33	3.33	1.003
T5F	5	7.10	26.1	3.33	5.66	1.006
T5F	6	6.00	24.9	3.46	5.28	1.009
T5F	7	4.93	41.7	7.35	9.59	1.015
T5F	10	1.98	25.6	11.5	6.82	1.008
T5F	24	0.95	10.2	9.09	3.33	1.006
T5F	48	0.73	20.9	27.5	0.7	1.015
T6F	0	1.02	8.63	8.21	2.00	1.005
T6F	1	1.88	11.8	5.76	5.0	1.002
T6F	2	2.27	13.1	5.30	2.50	1.004
T6F	3	2.93	18.8	6.20	2.89	1.008
T6F	4	2.82	24.2	7.69	3.54	1.019
T6F	5	3.26	31.0	9.24	3.60	1.016
T6F	6	3.65	28.0	7.41	3.52	1.008
T6F	7	3.90	52.5	13.3	7.47	1.013
T6F	10	2.21	23.2	9.62	5.01	1.013
T6F	24	1.04	14.5	12.8	6.90	1.012
T6F	48	1.15	14.2	11.8	7.61	1.010
T7F	0	0.26	12.7	44.5	4.0	1.017
T7F	1	0.60	13.2	21.7	5.0	1.002
T7F	2	1.39	18.6	23.4	5.00	1.002
T7F	3	0.67	18.3	25.4	2.00	1.005
T7F	4	0.65	15.6	20.7	2.84	1.016
T7F	5	0.94	20.0	19.9	3.01	1.013
T7F	6	1.00	29.2	25.8	3.01	1.012
T7F	7	0.62	33.7	51.3	4.69	1.020
T7F	10	0.24	11.1	42.6	4.62	1.022
T7F	24	0.23	9.03	38.5	2.5	1.004
T7F	48	0.25	8.10	31.8	3.36	1.008
T8F	0	0.38	5.00	22.5	5.00	1.002
T8F	1	0.60	5.00	10.0	5.00	1.002
T8F	2	0.67	5.00	11.7	5.00	1.002
T8F	3	0.88	14.9	16.5	6.04	1.008
T8F	4	1.33	18.2	14.1	6.64	1.02
T8F	5	1.47	35.2	22.7	9.15	1.022
T8F	6	1.39	35.4	26.5	8.98	1.022
T8F	7	0.88	22.7	27.7	0.83	1.012
T8F	10	0.54	13.1	26.4	7.76	1.015
T8F	24	0.37	6.21	15.9	4.35	1.009

T8F	48	0.51	6.43	11.5	5.18	1.004
T9F	0	0.94	11.3	12.9	6.30	1.009
T9F	1	2.24	14.2	5.0	5.00	1.002
T9F	2	2.89	21.4	8.19	2.50	1.004
T9F	3	2.89	23.4	8.02	5.36	1.014
T9F	4	3.11	47.9	15.8	9.89	1.004
T9F	5	4.53	31.2	8.38	4.72	1.009
T9F	6	3.81	33.7	9.43	4.98	1.005
T9F	7	4.64	54.6	11.1	7.17	1.010
T9F	10	1.26	45.7	28.2	19.80	1.026
T9F	24	0.86	11.2	12.8	5.99	1.017
T9F	48	0.79	9.59	12.0	4.61	1.015
T10F	0	0.86	25.9	25.2	11.7	1.024
T10F	1	1.49	15.9	10.6	2.5	1.004
T10F	2	1.80	19.3	10.1	4.48	1.009
T10F	3	1.97	27.3	12.1	5.23	1.015
T10F	4	2.70	41.6	15.0	6.29	1.012
T10F	5	3.17	55.1	18.2	9.32	1.019
T10F	6	2.54	35.1	12.5	7.19	1.018
T10F	7	2.06	35.2	16.8	11.7	1.019
T10F	10	1.12	13.5	11.9	7.25	1.019
T10F	24	0.55	29.1	40.1	18.6	1.026
T10F	48	0.60	27.2	34.3	12.1	1.024

8.18 Urinary T/E, T, E and 5α-DHT concentrations (ng/mL) for eugonadal males after 4 units

Appendix 8.18 Urinary T/E, T, E and 5α-DHT concentrations (ng/mL) for eugonadal males after 4 units

Sample	H after intake	T/E	T conc	E conc	5α-DHT conc	SG
T1M	0	0.09	2.78	26.9	2.16	1.012
T1M	1	0.11	1.67	23.2	1.67	1.006
T1M	2	0.14	2.67	17.3	2.51	1.008
T1M	3	0.12	2.37	17.5	2.56	1.009
T1M	4	0.11	4.21	33.9	5.03	1.022
T1M	5	0.11	4.46	30.6	5.08	1.024
T1M	6	0.13	3.15	20.7	3.14	1.021
T1M	7	0.14	3.17	24.8	3.75	1.020
T1M	10	0.12	3.01	19.4	2.30	1.025
T1M	24	0.10	2.00	30.4	2.00	1.005
T1M	48	0.13	1.43	16.8	1.43	1.007
T2M	0	1.00	58.0	56.0	7.53	1.01
T2M	1	1.07	79.1	73.8	7.81	1.011
T2M	2	1.26	56.3	44.5	6.03	1.004
T2M	3	1.08	68.9	64.0	7.51	1.011

T2M	4	0.95	53.4	53.0	6.94	1.010
T2M	5	0.99	76.1	70.6	8.86	1.012
T2M	6	1.09	83.8	69.7	9.00	1.016
T2M	7	1.09	50.3	46.2	7.03	1.004
T2M	10	1.22	42.2	35.5	5.70	1.005
T2M	24	1.27	69.4	54.8	7.25	1.02
T2M	48	0.96	36.1	36.8	5.36	1.006
T4M	0	0.43	26.0	6.25	5.32	1.007
T4M	1	0.55	16.9	30.1	2.50	1.004
T4M	2	0.57	34.8	7.36	4.35	1.019
T4M	3	0.46	23.4	50.4	4.38	1.011
T4M	4	0.43	32.8	65.0	6.82	1.012
T4M	5	0.39	29.6	70.9	6.01	1.011
T4M	6	0.39	29.5	69.8	6.13	1.007
T4M	7	0.41	39.8	88.4	9.80	1.016
T4M	10	0.42	23.9	56.6	5.59	1.005
T4M	24	0.33	34.3	100	6.68	1.010
T4M	48	0.31	32.8	101	6.06	1.010
T5M	0	0.23	13.4	50.1	4.01	1.026
T5M	1	0.26	5.11	17.0	1.67	1.006
T5M	2	0.28	6.05	17.0	2.59	1.017
T5M	3	0.31	4.74	13.7	1.93	1.011
T5M	4	0.27	6.89	22.9	2.61	1.011
T5M	5	0.23	5.61	20.6	2.55	1.019
T5M	6	0.24	4.38	16.2	1.89	1.016
T5M	7	0.30	7.27	21.2	2.62	1.020
T5M	10	0.29	6.07	17.8	2.22	1.023
T5M	24	0.31	12.9	34.9	3.07	1.021
T5M	48	0.29	10.3	25.6	2.30	1.022
T6M	0	0.06	3.49	44.7	1.83	1.011
T6M	1	0.06	1.99	27.4	0.91	1.011
T6M	2	0.07	2.00	21.1	2.00	1.005
T6M	3	0.09	2.50	28.1	2.50	1.004
T6M	4	0.07	2.73	33.2	1.94	1.015
T6M	5	0.08	2.00	24.2	2.00	1.005
T6M	6	0.11	2.50	23.8	2.50	1.004
T6M	7	0.08	3.41	44.4	1.11	1.009
T6M	10	0.04	2.40	40.8	1.48	1.021
T6M	24	0.04	3.17	60.8	2.18	1.024
T6M	48	0.05	1.87	31.9	0.67	1.015
T7M	0	1.11	65.4	58.2	6.45	1.022
T7M	1	1.32	46.6	35.1	5.00	1.002
T7M	2	1.33	52.5	38.8	3.79	1.008
T7M	3	1.12	45.5	38.0	5.42	1.009
T7M	4	1.01	31.3	31.5	4.57	1.005
T7M	5	0.97	31.5	30.2	3.60	1.007
T7M	6	0.97	18.4	19.3	2.44	1.010

T7M	7	0.95	27.8	27.5	3.54	1.010
T7M	10	1.10	45.7	37.4	5.11	1.019
T7M	24	0.82	37.1	40.2	5.00	1.017
T7M	48	0.67	44.9	61.9	6.84	1.017
T8M	0	2.21	81.4	33.3	8.12	1.018
T8M	1	3.11	60.5	19.7	2.50	1.004
T8M	2	3.37	81.8	24.6	7.33	1.011
T8M	3	2.72	48.1	18.3	8.83	1.013
T8M	4	2.90	54.1	19.4	6.88	1.016
T8M	5	2.82	52.2	17.3	6.95	1.016
T8M	6	2.22	44.0	18.2	5.11	1.007
T8M	7	2.45	59.7	21.3	5.90	1.007
T8M	10	3.02	38.9	11.9	4.15	1.011
T8M	24	1.35	33.9	24.8	3.15	1.006
T8M	48	1.69	39.6	21.1	3.54	1.007
T9M	0	1.53	84.9	53.7	15.6	1.010
T9M	1	1.62	52.3	25.2	47.7	1.005
T9M	2	1.99	191.5	89.9	27.6	1.005
T9M	3	1.94	33.7	16.6	7.60	1.009
T9M	4	1.99	32.3	15.4	8.26	1.007
T9M	5	1.81	27.3	14.9	6.98	1.008
T9M	6	1.87	28.5	13.6	7.60	1.009
T9M	7	1.88	29.8	15.8	10.8	1.012
T9M	10	2.46	20.5	7.91	7.88	1.020
T9M	24	1.78	41.5	22.7	8.02	1.014
T9M	48	2.23	22.7	10.2	7.48	1.006
T10M	0	0.13	4.79	38.0	3.09	1.029
T10M	1	0.14	3.45	25.7	1.92	1.024
T10M	2	0.16	2.84	19.3	2.56	1.024
T10M	3	0.16	3.46	23.6	3.40	1.025
T10M	4	0.18	2.70	17.5	2.80	1.022
T10M	5	0.15	2.94	23.2	2.02	1.022
T10M	6	0.14	2.53	24.9	2.60	1.023
T10M	7	0.14	2.55	23.3	1.80	1.022
T10M	10	0.14	1.20	17.0	1.80	1.021
T10M	24	0.14	3.75	25.2	1.98	1.022
T10M	48	0.11	3.14	27.4	1.58	1.014

8.19 Urinary T/E, T, E and 5α-DHT concentrations (ng/mL) for eugonadal males after 8 units

Appendix 8.19 Urinary T/E, T, E and 5α-DHT concentrations (ng/mL) for eugonadal males after 8 units

Sample	H after intake	T/E	T conc	E conc	5a-DHT conc	SG
T1M	0	0.09	3.43	35.3	2.93	1.017
T1M	1	0.16	10.00	46.5	10.0	1.001
T1M	2	0.11	1.67	28.9	1.67	1.006
T1M	3	0.10	2.91	25.5	1.43	1.007
T1M	4	0.13	3.92	29.0	1.67	1.006
T1M	5	0.11	4.35	35.3	3.42	1.006
T1M	6	0.10	3.68	29.7	2.62	1.017
T1M	7	0.10	2.66	24.7	2.59	1.010
T1M	10	0.09	3.23	33.1	3.76	1.010
T1M	24	0.08	2.74	31.0	2.05	1.012
T1M	48	0.08	3.03	33.7	2.70	1.010
T2M	0	0.90	42.7	45.9	5.00	1.002
T2M	1	1.13	59.2	46.4	7.27	1.004
T2M	2	1.33	49.2	35.7	5.00	1.002
T2M	3	1.41	59.8	39.4	6.08	1.004
T2M	4	1.38	92.9	59.6	9.38	1.008
T2M	5	1.29	102	65.9	9.10	1.014
T2M	6	1.21	105	73.6	11.2	1.015
T2M	7	1.05	69.9	55.1	12.8	1.013
T2M	10	1.01	65.2	57.4	10.8	1.006
T2M	24	0.80	60.8	65.9	8.67	1.008
T2M	48	0.85	63.0	67.1	8.49	1.013
T4M	0	0.31	30.0	79.4	9.16	1.021
T4M	1	0.51	12.5	23.8	3.33	1.003
T4M	2	0.55	27.3	42.5	4.17	1.006
T4M	3	0.61	24.2	33.9	4.04	1.009
T4M	4	0.58	34.7	53.4	5.12	1.011
T4M	5	0.55	40.9	66.0	5.85	1.014
T4M	6	0.53	31.8	52.6	5.38	1.011
T4M	7	0.42	28.9	60.7	7.51	1.014
T4M	10	0.53	33.0	58.5	9.59	1.008
T4M	24	0.32	31.7	88.8	7.45	1.006
T4M	48	0.34	34.7	82.3	8.21	1.006
T5M	0	0.24	5.95	23.4	2.55	1.011
T5M	1	0.26	5.00	11.9	5.0	1.002
T5M	2	0.32	2.50	8.9	2.50	1.004
T5M	3	0.30	3.17	10.2	1.67	1.006
T5M	4	0.24	3.35	11.7	1.61	1.013
T5M	5	0.31	3.58	11.1	1.64	1.014

T5M	6	0.28	4.82	14.3	2.06	1.012
T5M	7	0.25	5.89	17.4	2.39	1.01
T5M	10	0.29	3.33	11.1	3.33	1.003
T5M	24	0.20	3.54	13.5	2.34	1.012
T5M	48	0.23	4.79	17.4	1.85	1.014
T6M	0	0.07	2.81	46.0	0.50	1.020
T6M	1	0.06	2.50	28.4	2.50	1.004
T6M	2	0.12	5.00	25.7	5.00	1.002
T6M	3	0.10	5.00	26.6	5.00	1.002
T6M	4	0.09	2.50	32.3	2.50	1.004
T6M	5	0.07	1.11	35.6	2.47	1.009
T6M	6	0.06	1.63	29.7	4.36	1.013
T6M	7	0.08	3.27	37.6	3.79	1.013
T6M	10	0.06	2.00	40.5	6.02	1.005
T6M	24	0.05	1.43	43.4	3.41	1.007
T6M	48	0.04	2.35	49.0	0.63	1.016
T7M	0	0.97	48.7	37.7	5.27	1.019
T7M	1	1.02	39.0	31.8	5.03	1.004
T7M	2	1.45	51.5	32.2	3.33	1.003
T7M	3	1.45	122	73.8	15.7	1.003
T7M	4	1.29	71.3	42.1	5.72	1.018
T7M	5	1.16	67.3	53.4	7.16	1.022
T7M	6	1.39	101	68.6	6.43	1.02
T7M	7	1.10	41.3	41.5	6.38	1.005
T7M	10	0.99	19.2	19.9	2.50	1.004
T7M	24	0.76	25.6	33.6	2.50	1.004
T7M	48	0.65	31.3	50.1	5.01	1.007
T8M	0	1.61	54.2	38.2	7.42	1.012
T8M	1	2.49	68.6	28.3	3.33	1.003
T8M	2	3.03	87.2	32.4	5.98	1.006
T8M	3	3.18	136	43.5	8.65	1.018
T8M	4	3.18	155	47.1	9.62	1.022
T8M	5	3.11	157	48.8	10.0	1.022
T8M	6	2.98	138	47.5	11.1	1.018
T8M	7	2.71	94.3	37.9	11.8	1.006
T8M	10	2.77	89.2	34.8	8.95	1.014
T8M	24	1.27	43.0	34.5	5.00	1.002
T8M	48	1.84	52.6	29.7	6.60	1.004
T9M	0	1.47	65.1	40.9	10.3	1.019
T9M	1	1.79	42.8	26.4	4.29	1.009
T9M	2	2.09	139	69.4	13.3	1.004
T9M	3	2.16	44.8	22.2	4.05	1.007
T9M	4	2.01	46.9	23.0	4.62	1.007
T9M	5	2.81	61.2	22.4	4.65	1.014
T9M	6	2.57	67.4	27.8	6.31	1.014
T9M	7	2.50	91.8	36.2	8.70	1.016
T9M	10	1.71	36.9	21.6	7.83	1.015

T9M	24	1.27	49.2	40.9	11.8	1.017
T9M	48	1.41	89.4	60.9	18.8	1.02
T10M	0	0.07	4.77	59.8	3.07	1.030
T10M	1	0.15	2.50	9.3	2.50	1.004
T10M	2	0.19	3.33	15.7	3.33	1.003
T10M	3	0.16	2.50	16.2	2.50	1.004
T10M	4	0.11	2.14	20.7	1.87	1.014
T10M	5	0.11	2.68	23.3	1.87	1.018
T10M	6	0.13	2.63	21.9	1.95	1.016
T10M	7	0.13	2.30	18.0	2.31	1.013
T10M	10	0.13	2.50	24.4	2.50	1.004
T10M	24	0.09	2.06	22.2	2.23	1.019
T10M	48	0.12	1.25	21.9	1.25	1.008

8.20 Serum LH concentration for females after 4 units and after 8 units

Appendix 8.20 Serum LH concentration for females after 4 units and after 8 units

Female serum 4 units	LH concentration (IU/L)
T1F - S - 4U - CT	3.172
T1F - S - 4U - 1h	6.293
T1F - S - 4U - 2h	3.604
T1F - S - 4U - 3h	5.546
T1F - S - 4U - 4h	5.392
T1F - S - 4U - 5h	4.484
T1F - S - 4U - 6h	10.082
T1F - S - 4U - 24h	N/A
T1F - S - 4U - 48h	7.961
T2F - S - 4U - CT	6.739
T2F - S - 4U - 1h	4.911
T2F - S - 4U - 2h	4.66
T2F - S - 4U - 3h	3.674
T2F - S - 4U - 4h	2.245
T2F - S - 4U - 5h	5.712
T2F - S - 4U - 6h	2.786
T2F - S - 4U - 24h	3.947
T2F - S - 4U - 48h	4.038
T4F - S - 4U - CT	32.399
T4F - S - 4U - 1h	26.917
T4F - S - 4U - 2h	21.479
T4F - S - 4U - 3h	18.562
T4F - S - 4U - 4h	19.316
T4F - S - 4U - 5h	18.518
T4F - S - 4U - 6h	32.395
T4F - S - 4U - 24h	10.674

Female serum 8 units	LH concentration (IU/L)
T1F - S - 8U - CT	2.741
T1F - S - 8U - 1h	1.379
T1F - S - 8U - 2h	2.816
T1F - S - 8U - 3h	2.12
T1F - S - 8U - 4h	3.126
T1F - S - 8U - 5h	1.835
T1F - S - 8U - 6h	2.582
T1F - S - 8U - 24h	2.972
T1F - S - 8U - 48h	3.677
T2F - S - 8U - CT	7.97
T2F - S - 8U - 1h	5.638
T2F - S - 8U - 2h	7.543
T2F - S - 8U - 3h	4.246
T2F - S - 8U - 4h	2.653
T2F - S - 8U - 5h	1.963
T2F - S - 8U - 6h	1.797
T2F - S - 8U - 24h	6.681
T2F - S - 8U - 48h	7.917
T4F - S - 8U - CT	1.281
T4F - S - 8U - 1h	2.187
T4F - S - 8U - 2h	1.269
T4F - S - 8U - 3h	0.861
T4F - S - 8U - 4h	0.538
T4F - S - 8U - 5h	0.432
T4F - S - 8U - 6h	2.834
T4F - S - 8U - 24h	3.2

T4F - S - 4U - 48h	3.628
T5F - S - 4U - CT	23.033
T5F - S - 4U - 1h	17.893
T5F - S - 4U - 2h	11.549
T5F - S - 4U - 3h	11.16
T5F - S - 4U - 4h	15.721
T5F - S - 4U - 5h	11.751
T5F - S - 4U - 6h	14.095
T5F - S - 4U - 24h	5.726
T5F - S - 4U - 48h	4.466
T6F - S - 4U - CT	12.368
T6F - S - 4U - 1h	4.911
T6F - S - 4U - 2h	5.872
T6F - S - 4U - 3h	3.217
T6F - S - 4U - 4h	3.069
T6F - S - 4U - 5h	2.489
T6F - S - 4U - 6h	2.335
T6F - S - 4U - 24h	3.356
T6F - S - 4U - 48h	4.665
T7F - S - 4U - CT	9.708
T7F - S - 4U - 1h	10.287
T7F - S - 4U - 2h	8.927
T7F - S - 4U - 3h	8.596
T7F - S - 4U - 4h	8.843
T7F - S - 4U - 5h	9.493
T7F - S - 4U - 6h	6.865
T7F - S - 4U - 24h	23.006
T7F - S - 4U - 48h	23.051
T8F - S - 4U - CT	12.098
T8F - S - 4U - 1h	13.897
T8F - S - 4U - 2h	9.624
T8F - S - 4U - 3h	15.323
T8F - S - 4U - 4h	18.518
T8F - S - 4U - 5h	20.648
T8F - S - 4U - 6h	12.85
T8F - S - 4U - 24h	8.154
T8F - S - 4U - 48h	8.913
T9F - S - 4U - CT	3.708
T9F - S - 4U - 1h	2.326
T9F - S - 4U - 2h	4.313
T9F - S - 4U - 3h	4.365
T9F - S - 4U - 4h	4.277
T9F - S - 4U - 5h	4.248
T9F - S - 4U - 6h	5.762
T9F - S - 4U - 24h	6.151
T9F - S - 4U - 48h	10.462
T10F - S - 4U -CT	4.994
T10F - S - 4U - 1h	4.656
T10F - S - 4U - 2h	4.134
T10F - S - 4U - 3h	3.396
T10F - S - 4U - 4h	3.605

T4F - S - 8U - 48h	2.386
T5F - S - 8U - CT	2.556
T5F - S - 8U - 1h	1.801
T5F - S - 8U - 2h	1.054
T5F - S - 8U - 3h	1.764
T5F - S - 8U - 4h	0.92
T5F - S -8U - 5h	1.185
T5F - S - 8U - 6h	0.51
T5F - S - 8U - 24h	1.757
T5F - S - 8U - 48h	2.397
T6F - S - 8U - CT	5.513
T6F - S - 8U - 1h	5.136
T6F - S - 8U - 2h	3.337
T6F - S - 8U - 3h	5.654
T6F - S - 8U - 4h	8.058
T6F - S - 8U - 5h	7.495
T6F - S - 8U - 6h	7.635
T6F - S - 8U - 24h	6.686
T6F - S - 8U - 48h	5.863
T7F - S - 8U - CT	6.266
T7F - S - 8U - 1h	2.657
T7F - S - 8U - 2h	5.872
T7F - S - 8U - 3h	3.263
T7F - S - 8U - 4h	6.293
T7F - S - 8U - 5h	7.195
T7F - S - 8U - 6h	5.605
T7F - S - 8U - 24h	5.555
T7F - S - 8U - 48h	4.5
T8F - S - 8U - CT	3.626
T8F - S - 8U - 1h	6.925
T8F - S - 8U - 2h	4.044
T8F - S - 8U - 3h	3.077
T8F - S - 8U - 4h	7.575
T8F - S - 8U - 5h	6.779
T8F - S - 8U - 6h	4.353
T8F - S - 8U - 24h	2.493
T8F - S - 8U - 48h	7.904
T9F - S - 8U - CT	5.555
T9F - S - 8U - 1h	5.122
T9F - S - 8U - 2h	7.431
T9F - S - 8U - 3h	4.039
T9F - S - 8U - 4h	4.788
T9F - S - 8U - 5h	5.872
T9F - S - 8U - 6h	5.365
T9F - S - 8U - 24h	6.456
T9F - S - 8U - 48h	10.793
T10F - S - 8U -CT	8.366
T10F - S - 8U - 1h	5.665
T10F - S - 8U - 2h	6.438
T10F - S - 8U - 3h	5.925
T10F - S - 8U - 4h	6.385

T10F - S - 4U - 5h	4.574
T10F - S - 4U - 6h	4.656
T10F - S - 4U-24h	6.084
T10F - S - 4U-48h	5.934

T10F - S - 8U - 5h	7.986
T10F - S - 8U - 6h	7.796
T10F - S – 8U-24h	5.05
T10F - S - 8U-48h	23.557

8.21 Urinary LH concentration for females after 4 and after 8 units

Appendix 8.21 Urinary LH concentration for females after 4 and after 8 units

Female urine 4 units	LH adjusted concentration (IU/L)
T1F - U - 4U - CT	12.183
T1F - U - 4U - 1h	6.240
T1F - U - 4U - 2h	3.955
T1F - U - 4U - 3h	6.088
T1F - U - 4U - 4h	5.290
T1F - U - 4U - 5h	8.445
T1F - U - 4U - 6h	6.051
T1F - U - 4U - 7h	13.895
T1F - U - 4U - 10h	5.977
T1F - U - 4U - 24h	8.826
T1F - U - 4U - 48h	4.027
T1F - U - 4U - 72h	5.431
T2F - U - 4U - CT	6.547
T2F - U - 4U - 1h	3.430
T2F - U - 4U - 2h	4.188
T2F - U - 4U - 3h	4.558
T2F - U - 4U - 4h	4.958
T2F - U - 4U - 5h	6.528
T2F - U - 4U - 6h	5.996
T2F - U - 4U - 7h	3.271
T2F - U - 4U - 10h	4.336
T2F - U - 4U - 24h	4.174
T2F - U - 4U - 48h	5.154
T2F - U - 4U - 72h	6.507
T3F - U - 4U - CT	14.955
T3F - U - 4U - 1h	11.667
T3F - U - 4U - 2h	2.412
T3F - U - 4U - 3h	6.660
T3F - U - 4U - 4h	10.760
T3F - U - 4U - 5h	6.532
T3F - U - 4U - 6h	7.062
T3F - U - 4U - 7h	8.431
T3F - U - 4U - 10h	16.866

Female urine 8 units	LH adjusted concentration (IU/L)
T1F - U - 8U - CT	5.471
T1F - U - 8U - 1h	0.700
T1F - U - 8U - 2h	2.880
T1F - U - 8U - 3h	2.503
T1F - U - 8U - 4h	3.228
T1F - U - 8U - 5h	2.720
T1F - U - 8U - 6h	3.064
T1F - U - 8U - 7h	3.380
T1F - U - 8U - 10h	4.696
T1F - U - 8U - 24h	4.152
T1F - U - 8U - 48h	2.573
T1F - U - 8U - 72h	-0.005
T2F - U - 8U - CT	11.878
T2F - U - 8U - 1h	2.927
T2F - U - 8U - 2h	3.853
T2F - U - 8U - 3h	7.445
T2F - U - 8U - 4h	5.133
T2F - U - 8U - 5h	3.878
T2F - U - 8U - 6h	2.957
T2F - U - 8U - 7h	8.557
T2F - U - 8U - 10h	3.913
T2F - U - 8U - 24h	5.720
T2F - U - 8U - 48h	12.328
T2F - U - 8U - 72h	10.890
T3F - U - 8U - CT	20.240
T3F - U - 8U - 1h	5.713
T3F - U - 8U - 2h	6.250
T3F - U - 8U - 3h	3.190
T3F - U - 8U - 4h	2.870
T3F - U - 8U - 5h	3.253
T3F - U - 8U - 6h	4.384
T3F - U - 8U - 7h	4.520
T3F - U - 8U - 10h	6.353

T3F - U - 4U - 24h	17.830
T3F - U - 4U - 48h	55.820
T3F - U - 4U - 72h	2.140
T4F - U - 4U - CT	38.737
T4F - U - 4U - 1h	18.908
T4F - U - 4U - 2h	21.380
T4F - U - 4U - 3h	20.094
T4F - U - 4U - 4h	19.816
T4F - U - 4U - 5h	27.664
T4F - U - 4U - 6h	25.324
T4F - U - 4U - 7h	70.171
T4F - U - 4U - 10h	72.127
T4F - U - 4U - 24h	112.362
T4F - U - 4U - 48h	10.160
T4F - U - 4U - 72h	8.727
T5F - U - 4U - CT	63.627
T5F - U - 4U - 1h	10.000
T5F - U - 4U - 2h	14.760
T5F - U - 4U - 3h	17.900
T5F - U - 4U - 4h	19.864
T5F - U - 4U - 5h	25.080
T5F - U - 4U - 6h	31.107
T5F - U - 4U - 7h	43.558
T5F - U - 4U - 10h	25.720
T5F - U - 4U - 24h	4.440
T5F - U - 4U - 48h	24.860
T5F - U - 4U - 72h	8.760
T6F - U - 4U - CT	3.080
T6F - U - 4U - 1h	1.400
T6F - U - 4U - 2h	1.238
T6F - U - 4U - 3h	2.325
T6F - U - 4U - 4h	2.390
T6F - U - 4U - 5h	1.841
T6F - U - 4U - 6h	2.229
T6F - U - 4U - 7h	2.601
T6F - U - 4U - 10h	1.478
T6F - U - 4U - 24h	1.971
T6F - U - 4U - 48h	1.847
T6F - U - 4U - 72h	2.353
T7F - U - 4U - CT	2.063
T7F - U - 4U - 1h	1.520
T7F - U - 4U - 2h	1.128
T7F - U - 4U - 3h	1.700
T7F - U - 4U - 4h	4.094

T3F - U - 8U - 24h	14.922
T3F - U - 8U - 48h	11.591
T3F - U - 8U - 72h	8.072
T4F - U - 8U - CT	2.223
T4F - U - 8U - 1h	0.953
T4F - U - 8U - 2h	0.700
T4F - U - 8U - 3h	1.337
T4F - U - 8U - 4h	1.082
T4F - U - 8U - 5h	1.059
T4F - U - 8U - 6h	3.040
T4F - U - 8U - 7h	3.220
T4F - U - 8U - 10h	2.160
T4F - U - 8U - 24h	3.879
T4F - U - 8U - 48h	3.410
T4F - U - 8U - 72h	2.980
T5F - U - 8U - CT	4.828
T5F - U - 8U - 1h	1.873
T5F - U - 8U - 2h	1.820
T5F - U - 8U - 3h	0.700
T5F - U - 8U - 4h	2.193
T5F - U - 8U - 5h	2.897
T5F - U - 8U - 6h	2.933
T5F - U - 8U - 7h	3.931
T5F - U - 8U - 10h	2.013
T5F - U - 8U - 24h	1.637
T5F - U - 8U - 48h	8.057
T5F - U - 8U - 72h	2.520
T6F - U - 8U - CT	2.864
T6F - U - 8U - 1h	0.700
T6F - U - 8U - 2h	0.350
T6F - U - 8U - 3h	1.900
T6F - U - 8U - 4h	4.246
T6F - U - 8U - 5h	4.885
T6F - U - 8U - 6h	4.355
T6F - U - 8U - 7h	5.872
T6F - U - 8U - 10h	5.106
T6F - U - 8U - 24h	4.118
T6F - U - 8U - 48h	5.776
T6F - U - 8U - 72h	8.268
T7F - U - 8U - CT	13.991
T7F - U - 8U - 1h	0.700
T7F - U - 8U - 2h	1.780
T7F - U - 8U - 3h	3.048
T7F - U - 8U - 4h	3.906

T7F - U - 4U - 5h	1.040
T7F - U - 4U - 6h	2.807
T7F - U - 4U - 7h	2.687
T7F - U - 4U - 10h	2.245
T7F - U - 4U - 24h	18.917
T7F - U - 4U - 48h	22.743
T7F - U - 4U - 72h	23.417
T8F - U - 4U - CT	2.520
T8F - U - 4U - 1h	3.110
T8F - U - 4U - 2h	3.324
T8F - U - 4U - 3h	4.786
T8F - U - 4U - 4h	3.558
T8F - U - 4U - 5h	10.723
T8F - U - 4U - 6h	5.858
T8F - U - 4U - 7h	4.993
T8F - U - 4U - 10h	4.703
T8F - U - 4U - 24h	4.709
T8F - U - 4U - 48h	4.765
T8F - U - 4U - 72h	3.876
T9F - U - 4U - CT	6.120
T9F - U - 4U - 1h	1.027
T9F - U - 4U - 2h	1.772
T9F - U - 4U - 3h	3.131
T9F - U - 4U - 4h	1.983
T9F - U - 4U - 5h	2.475
T9F - U - 4U - 6h	1.934
T9F - U - 4U - 7h	2.227
T9F - U - 4U - 10h	3.056
T9F - U - 4U - 24h	3.809
T9F - U - 4U - 48h	5.568
T9F - U - 4U - 72h	6.861
T10F - U - 4U-CT	1.888
T10F - U - 4U - 1h	0.993
T10F - U - 4U - 2h	0.516
T10F - U - 4U - 3h	0.600
T10F - U - 4U - 4h	1.018
T10F - U - 4U - 5h	0.884
T10F - U - 4U - 6h	0.829
T10F - U - 4U - 7h	0.980
T10F - U - 4U-10h	1.691
T10F - U - 4U-24h	1.973
T10F - U - 4U-48h	1.109
T10F - U - 4U-72h	2.809

T7F - U - 8U - 5h	4.718
T7F - U - 8U - 6h	5.718
T7F - U - 8U - 7h	10.882
T7F - U - 8U - 10h	8.533
T7F - U - 8U - 24h	4.375
T7F - U - 8U - 48h	4.830
T7F - U - 8U - 72h	5.072
T8F - U - 8U - CT	3.870
T8F - U - 8U - 1h	1.860
T8F - U - 8U - 2h	3.760
T8F - U - 8U - 3h	4.720
T8F - U - 8U - 4h	4.989
T8F - U - 8U - 5h	6.916
T8F - U - 8U - 6h	7.997
T8F - U - 8U - 7h	7.685
T8F - U - 8U - 10h	8.539
T8F - U - 8U - 24h	5.573
T8F - U - 8U - 48h	6.970
T8F - U - 8U - 72h	5.710
T9F - U - 8U - CT	11.409
T9F - U - 8U - 1h	2.850
T9F - U - 8U - 2h	3.795
T9F - U - 8U - 3h	5.333
T9F - U - 8U - 4h	10.855
T9F - U - 8U - 5h	6.064
T9F - U - 8U - 6h	7.088
T9F - U - 8U - 7h	10.582
T9F - U - 8U - 10h	15.600
T9F - U - 8U - 24h	9.714
T9F - U - 8U - 48h	10.068
T9F - U - 8U - 72h	12.004
T10F - U - 8U-CT	8.705
T10F - U - 8U - 1h	2.705
T10F - U - 8U - 2h	2.449
T10F - U - 8U - 3h	2.819
T10F - U - 8U - 4h	2.788
T10F - U - 8U - 5h	3.600
T10F - U - 8U - 6h	3.781
T10F - U - 8U - 7h	4.805
T10F - U - 8U-10h	8.377
T10F - U - 8U-24h	38.135
T10F - U - 8U-48h	19.593
T10F - U - 8U-72h	14.852

8.22 Serum LH concentration for eugonadal males after 4 and after 8 units

Appendix 8.22 Serum LH concentration for eugonadal males after 4 and after 8 units

Male serum 4 units	LH concentration (IU/L)
T1M - S - 4U - CT	1.956
T1M - S - 4U - 1h	2.707
T1M - S - 4U - 2h	1.701
T1M - S - 4U - 3h	4.092
T1M - S - 4U - 4h	2.848
T1M - S - 4U - 5h	2.33
T1M - S - 4U - 6h	1.402
T1M - S - 4U - 24h	3.528
T1M - S - 4U - 48h	3.178
T2M - S - 4U - CT	2.738
T2M - S - 4U - 1h	3.151
T2M - S - 4U - 2h	4.2
T2M - S - 4U - 3h	2.676
T2M - S - 4U - 4h	3.188
T2M - S - 4U - 5h	3.445
T2M - S - 4U - 6h	2.737
T2M - S - 4U - 24h	N/A
T2M - S - 4U - 48h	3.985
T4M - S - 4U - CT	1.17
T4M - S - 4U - 1h	2.525
T4M - S - 4U - 2h	2.146
T4M - S - 4U - 3h	1.596
T4M - S - 4U - 4h	3.413
T4M - S - 4U - 5h	2.638
T4M - S - 4U - 6h	1.66
T4M - S - 4U - 24h	1.906
T4M - S - 4U - 48h	3.099
T5M - S - 4U - CT	7.436
T5M - S - 4U - 1h	5.932
T5M - S - 4U - 2h	5.406
T5M - S - 4U - 3h	8.237
T5M - S - 4U - 4h	8.736
T5M - S - 4U - 5h	5.694
T5M - S - 4U - 6h	5.726
T5M - S - 4U - 24h	7.085
T5M - S - 4U - 48h	5.627
T6M - S - 4U -CT	10.767
T6M - S - 4U - 1h	12.027
T6M - S - 4U - 2h	10.801
T6M - S - 4U - 3h	14.455
T6M - S - 4U - 4h	14.184
T6M - S - 4U - 5h	11.807
T6M - S - 4U - 6h	14.754

Male serum 8 units	LH concentration (IU/L)
T1M - S - 8U - CT	1.512
T1M - S - 8U - 1h	1.245
T1M - S - 8U - 2h	2.526
T1M - S - 8U - 3h	2.224
T1M - S - 8U - 4h	1.566
T1M - S - 8U - 5h	1.091
T1M - S - 8U - 6h	2.33
T1M - S - 8U - 24h	2.296
T1M - S - 8U - 48h	1.362
T2M - S - 8U - CT	2.645
T2M - S - 8U - 1h	3.063
T2M - S - 8U - 2h	3.142
T2M - S - 8U - 3h	2.014
T2M - S - 8U - 4h	2.257
T2M - S - 8U - 5h	1.657
T2M - S - 8U - 6h	1.99
T2M - S - 8U - 24h	3.784
T2M - S - 8U - 48h	2.36
T4M - S - 8U - CT	1.634
T4M - S - 8U - 1h	2.87
T4M - S - 8U - 2h	1.73
T4M - S - 8U - 3h	2.791
T4M - S - 8U - 4h	2.866
T4M - S - 8U - 5h	2.088
T4M - S - 8U - 6h	1.443
T4M - S - 8U - 24h	2.498
T4M - S - 8U - 48h	2.12
T5M - S - 8U - CT	5.351
T5M - S - 8U - 1h	2.923
T5M - S - 8U - 2h	2.634
T5M - S - 8U - 3h	6.378
T5M - S - 8U - 4h	4.719
T5M - S - 8U - 5h	9.605
T5M - S - 8U - 6h	6.95
T5M - S - 8U - 24h	7.203
T5M - S - 8U - 48h	6.449
T6M - S - 8U - CT	10.158
T6M - S - 8U - 1h	13.121
T6M - S - 8U - 2h	10.376
T6M - S - 8U - 3h	12.834
T6M - S - 8U - 4h	10.637
T6M - S - 8U - 5h	15.457
T6M - S - 8U - 6h	11.732

T6M - S - 4U - 24h	11.068
T6M - S - 4U - 48h	10.999
T7M - S - 4U - CT	3.594
T7M - S - 4U - 1h	2.431
T7M - S - 4U - 2h	1.931
T7M - S - 4U - 3h	1.934
T7M - S - 4U - 4h	1.375
T7M - S - 4U - 5h	1.82
T7M - S - 4U - 6h	1.693
T7M - S - 4U - 24h	3.701
T7M - S - 4U - 48h	4.041
T8M - S - 4U - CT	4.068
T8M - S - 4U - 1h	3.448
T8M - S - 4U - 2h	2.759
T8M - S - 4U - 3h	2.526
T8M - S - 4U - 4h	5.292
T8M - S - 4U - 5h	6.637
T8M - S - 4U - 6h	4.573
T8M - S - 4U - 24h	5.226
T8M - S - 4U - 48h	8.097
T9M - S - 4U - CT	2.728
T9M - S - 4U - 1h	3.192
T9M - S - 4U - 2h	2.702
T9M - S - 4U - 3h	2.313
T9M - S - 4U - 4h	1.921
T9M - S - 4U - 5h	1.579
T9M - S - 4U - 6h	1.536
T9M - S - 4U - 24h	3.448
T9M - S - 4U - 48h	1.883
T10M - S - 4U - CT	4.434
T10M - S - 4U - 1h	2.602
T10M - S - 4U - 2h	3.243
T10M - S - 4U - 3h	2.412
T10M - S - 4U - 4h	3.081
T10M - S - 4U - 5h	2.847
T10M - S - 4U - 6h	3.119
T10M - S - 4U - 24h	4.99
T10M - S - 4U - 48h	5.126

T6M - S - 8U - 24h	12.041
T6M - S - 8U - 48h	10.683
T7M - S - 8U - CT	2.447
T7M - S - 8U - 1h	1.541
T7M - S - 8U - 2h	1.438
T7M - S - 8U - 3h	3.484
T7M - S - 8U - 4h	3.2
T7M - S - 8U - 5h	3.44
T7M - S - 8U - 6h	3.07
T7M - S -8U - 24h	2.473
T7M - S - 8U - 48h	3.224
T8M - S - 8U - CT	3.54
T8M - S - 8U - 1h	7.543
T8M - S - 8U - 2h	4.099
T8M - S - 8U - 3h	4.464
T8M - S - 8U - 4h	6.718
T8M - S - 8U - 5h	4.282
T8M - S - 8U - 6h	3.732
T8M - S - 8U - 24h	4.447
T8M - S - 8U - 48h	3.169
T9M - S - 8U - CT	3.989
T9M - S - 8U - 1h	6.408
T9M - S - 8U - 2h	3.503
T9M - S - 8U - 3h	3.576
T9M - S - 8U - 4h	4.655
T9M - S - 8U - 5h	5.419
T9M - S - 8U - 6h	5.817
T9M - S - 8U - 24h	3.057
T9M - S - 8U - 48h	3.67
T10M - S - 8U-CT	3.794
T10M - S - 8U - 1h	3.394
T10M - S - 8U - 2h	4.905
T10M - S - 8U - 3h	4.454
T10M - S - 8U - 4h	4.662
T10M - S - 8U - 5h	3.508
T10M - S - 8U - 6h	4.086
T10M - S - 8U - 24h	3.314
T10M - S - 8U - 48h	3.061

8.23 Urinary LH concentration for eugonadal males after 4 and after 8 units

Appendix 8.23 Urinary LH concentration for eugonadal males after 4 and after 8 units

Male urine 4 units	LH adjusted concentration (IU/L)
T1M - U - 4U - CT	3.758
T1M - U - 4U - 1h	2.810

Male urine 8 units	LH adjusted concentration (IU/L)
T1M - U - 8U - CT	2.700
T1M - U - 8U - 1h	0.070

T1M - U - 4U - 2h	2.358
T1M - U - 4U - 3h	3.691
T1M - U - 4U - 4h	6.741
T1M - U - 4U - 5h	5.569
T1M - U - 4U - 6h	4.793
T1M - U - 4U - 7h	6.709
T1M - U - 4U - 10h	5.353
T1M - U - 4U - 24h	3.232
T1M - U - 4U - 48h	2.154
T1M - U - 4U - 72h	3.829
T2M - U - 4U - CT	4.242
T2M - U - 4U - 1h	4.344
T2M - U - 4U - 2h	2.055
T2M - U - 4U - 3h	3.636
T2M - U - 4U - 4h	3.768
T2M - U - 4U - 5h	3.958
T2M - U - 4U - 6h	4.390
T2M - U - 4U - 7h	1.625
T2M - U - 4U - 10h	1.764
T2M - U - 4U - 24h	5.315
T2M - U - 4U - 48h	2.357
T2M - U - 4U - 72h	4.356
T4M - U - 4U - CT	1.720
T4M - U - 4U - 1h	2.800
T4M - U - 4U - 2h	3.108
T4M - U - 4U - 3h	2.142
T4M - U - 4U - 4h	3.755
T4M - U - 4U - 5h	3.236
T4M - U - 4U - 6h	2.971
T4M - U - 4U - 7h	2.541
T4M - U - 4U - 10h	0.812
T4M - U - 4U - 24h	4.850
T4M - U - 4U - 48h	6.240
T4M - U - 4U - 72h	3.853
T5M - U - 4U - CT	25.857
T5M - U - 4U - 1h	6.960
T5M - U - 4U - 2h	8.818
T5M - U - 4U - 3h	11.851
T5M - U - 4U - 4h	19.015
T5M - U - 4U - 5h	13.308
T5M - U - 4U - 6h	12.739
T5M - U - 4U - 7h	16.910
T5M - U - 4U - 10h	15.775
T5M - U - 4U - 24h	23.361

T1M - U - 8U - 2h	2.537
T1M - U - 8U - 3h	2.894
T1M - U - 8U - 4h	2.250
T1M - U - 8U - 5h	1.687
T1M - U - 8U - 6h	3.313
T1M - U - 8U - 7h	2.958
T1M - U - 8U - 10h	2.208
T1M - U - 8U - 24h	3.970
T1M - U - 8U - 48h	2.272
T1M - U - 8U - 72h	2.961
T2M - U - 8U - CT	0.700
T2M - U - 8U - 1h	2.800
T2M - U - 8U - 2h	0.700
T2M - U - 8U - 3h	2.055
T2M - U - 8U - 4h	3.215
T2M - U - 8U - 5h	3.356
T2M - U - 8U - 6h	4.251
T2M - U - 8U - 7h	4.360
T2M - U - 8U - 10h	3.247
T2M - U - 8U - 24h	4.455
T2M - U - 8U - 48h	4.643
T2M - U - 8U - 72h	3.172
T4M - U - 8U - CT	3.018
T4M - U - 8U - 1h	1.467
T4M - U - 8U - 2h	2.487
T4M - U - 8U - 3h	3.591
T4M - U - 8U - 4h	4.456
T4M - U - 8U - 5h	2.904
T4M - U - 8U - 6h	2.407
T4M - U - 8U - 7h	3.144
T4M - U - 8U - 10h	2.230
T4M - U - 8U - 24h	2.453
T4M - U - 8U - 48h	3.743
T4M - U - 8U - 72h	0.923
T5M - U - 8U - CT	12.678
T5M - U - 8U - 1h	2.170
T5M - U - 8U - 2h	3.585
T5M - U - 8U - 3h	6.140
T5M - U - 8U - 4h	11.598
T5M - U - 8U - 5h	8.786
T5M - U - 8U - 6h	13.778
T5M - U - 8U - 7h	9.258
T5M - U - 8U - 10h	3.367
T5M - U - 8U - 24h	12.908

T5M - U - 4U - 48h	17.721
T5M - U - 4U - 72h	17.564
T6M - U - 4U - CT	6.218
T6M - U - 4U - 1h	5.424
T6M - U - 4U - 2h	3.544
T6M - U - 4U - 3h	4.130
T6M - U - 4U - 4h	4.379
T6M - U - 4U - 5h	3.408
T6M - U - 4U - 6h	4.080
T6M - U - 4U - 7h	8.998
T6M - U - 4U - 10h	5.940
T6M - U - 4U - 24h	6.256
T6M - U - 4U - 48h	4.956
T6M - U - 4U - 72h	10.398
T7M - U - 4U - CT	4.656
T7M - U - 4U - 1h	1.820
T7M - U - 4U - 2h	2.763
T7M - U - 4U - 3h	2.476
T7M - U - 4U - 4h	1.756
T7M - U - 4U - 5h	1.637
T7M - U - 4U - 6h	1.320
T7M - U - 4U - 7h	2.678
T7M - U - 4U - 10h	2.934
T7M - U - 4U - 24h	3.549
T7M - U - 4U - 48h	4.319
T7M - U - 4U - 72h	3.754
T8M - U - 4U - CT	4.904
T8M - U - 4U - 1h	1.810
T8M - U - 4U - 2h	2.558
T8M - U - 4U - 3h	2.298
T8M - U - 4U - 4h	2.986
T8M - U - 4U - 5h	3.650
T8M - U - 4U - 6h	2.371
T8M - U - 4U - 7h	2.326
T8M - U - 4U - 10h	1.925
T8M - U - 4U - 24h	1.623
T8M - U - 4U - 48h	1.860
T8M - U - 4U - 72h	0.000
T9M - U - 4U - CT	15.834
T9M - U - 4U - 1h	4.664
T9M - U - 4U - 2h	15.144
T9M - U - 4U - 3h	1.847
T9M - U - 4U - 4h	2.406
T9M - U - 4U - 5h	3.288

T5M - U - 8U - 48h	13.591
T5M - U - 8U - 72h	7.247
T6M - U - 8U - CT	5.882
T6M - U - 8U - 1h	2.815
T6M - U - 8U - 2h	1.520
T6M - U - 8U - 3h	0.700
T6M - U - 8U - 4h	3.110
T6M - U - 8U - 5h	3.238
T6M - U - 8U - 6h	3.837
T6M - U - 8U - 7h	4.582
T6M - U - 8U - 10h	2.848
T6M - U - 8U - 24h	6.997
T6M - U - 8U - 48h	4.781
T6M - U - 8U - 72h	4.093
T7M - U - 8U - CT	3.031
T7M - U - 8U - 1h	1.880
T7M - U - 8U - 2h	2.747
T7M - U - 8U - 3h	12.767
T7M - U - 8U - 4h	6.293
T7M - U - 8U - 5h	5.039
T7M - U - 8U - 6h	5.338
T7M - U - 8U - 7h	1.860
T7M - U - 8U - 10h	1.160
T7M - U - 8U - 24h	1.980
T7M - U - 8U - 48h	2.980
T7M - U - 8U - 72h	2.435
T8M - U - 8U - CT	3.620
T8M - U - 8U - 1h	2.213
T8M - U - 8U - 2h	2.430
T8M - U - 8U - 3h	3.832
T8M - U - 8U - 4h	2.684
T8M - U - 8U - 5h	2.525
T8M - U - 8U - 6h	3.392
T8M - U - 8U - 7h	2.397
T8M - U - 8 - 10h	3.253
T8M - U - 8U - 24h	1.900
T8M - U - 8U - 48h	1.415
T8M - U - 8U - 72h	2.380
T9M - U - 8U - CT	4.427
T9M - U - 8U - 1h	1.242
T9M - U - 8U - 2h	9.780
T9M - U - 8U - 3h	1.669
T9M - U - 8U - 4h	1.974
T9M - U - 8U - 5h	1.967

T9M - U - 4U - 6h	4.071
T9M - U - 4U - 7h	8.257
T9M - U - 4U - 10h	5.423
T9M - U - 4U - 24h	6.343
T9M - U - 4U - 48h	4.237
T9M - U - 4U - 72h	1.750
T10M - U - 4U - CT	5.637
T10M - U - 4U - 1h	3.639
T10M - U - 4U - 2h	2.277
T10M - U - 4U - 3h	2.401
T10M - U - 4U - 4h	2.644
T10M - U - 4U - 5h	3.247
T10M - U - 4U - 6h	3.674
T10M - U - 4U - 7h	3.795
T10M - U - 4U -10h	2.031
T10M - U - 4U -24h	4.638
T10M - U - 4U -48h	3.224
T10M - U - 4U -72h	4.107

T9M - U - 8U - 6h	2.431
T9M - U - 8U - 7h	10.355
T9M - U - 8U - 10h	5.537
T9M - U - 8U - 24h	10.292
T9M - U - 8U - 48h	14.525
T9M - U - 8U - 72h	10.167
T10M - U - 8U -CT	5.857
T10M - U - 8U - 1h	1.385
T10M - U - 8U - 2h	3.793
T10M - U - 8U - 3h	1.815
T10M - U - 8U - 4h	3.511
T10M - U - 8U - 5h	3.291
T10M - U - 8U - 6h	2.694
T10M - U - 8U - 7h	2.792
T10M - U - 8U-10h	3.385
T10M - U - 8U-24h	2.962
T10M - U - 8U-48h	2.300
T10M - U - 8U-72h	2.274

8.24 Serum alcohol concentrations in eugonadal males, after 4 units of alcohol

Appendix 8.24 Serum alcohol concentrations in males, after 4 units of alcohol

Male serum 4 units	Serum alcohol concentration g/L	Serum alcohol concentration mg%
T1M - S - 4U - CT	0	0.00
T1M - S - 4U - 1h	0.23	23.00
T1M - S - 4U - 2h	0.14	14.00
T1M - S - 4U - 3h	0.05	5.00
T1M - S - 4U - 4h	0.01	1.00
T1M - S - 4U - 5h	0.01	1.00
T1M - S - 4U - 6h	0	0.00
T1M - S - 4U - 24h	0	0.00
T2M - S - 4U - CT	0	0.00
T2M - S - 4U - 1h	0.25	25.00
T2M - S - 4U - 2h	0.15	15.00
T2M - S - 4U - 3h	0.07	7.00
T2M - S - 4U - 4h	0.03	3.00
T2M - S - 4U - 5h	0.01	1.00
T2M - S - 4U - 6h	0.01	1.00
T4M - S - 4U - CT	0	0.00
T4M - S - 4U - 1h	0.25	25.00
T4M - S - 4U - 2h	0.1	10.00
T4M - S - 4U - 3h	0.02	2.00
T4M - S - 4U - 4h	0.01	1.00
T4M - S - 4U - 5h	0	0.00

T4M - S - 4U - 6h	0	0.00
T4M - S - 4U - 24h	0	0.00
T5M - S - 4U - CT	0	0.00
T5M - S - 4U - 1h	0.28	28.00
T5M - S - 4U - 2h	0.14	14.00
T5M - S - 4U - 3h	0.06	6.00
T5M - S - 4U - 4h	0.02	2.00
T5M - S - 4U - 5h	0.01	1.00
T5M - S - 4U - 6h	0.01	1.00
T5M - S - 4U - 24h	0	0.00
T6M - S - 4U -CT	0	0.00
T6M - S - 4U - 1h	0.4	40.00
T6M - S - 4U - 2h	0.29	29.00
T6M - S - 4U - 3h	0.18	18.00
T6M - S - 4U - 4h	0.07	7.00
T6M - S - 4U - 5h	0.03	3.00
T6M - S - 4U - 6h	0.01	1.00
T6M - S - 4U - 24h	0	0.00
T7M - S - 4U - CT	0	0.00
T7M - S - 4U - 1h	0.32	32.00
T7M - S - 4U - 2h	0.13	13.00
T7M - S - 4U - 3h	0.04	4.00
T7M - S - 4U - 4h	0.01	1.00
T7M - S - 4U - 5h	0	0.00
T7M - S - 4U - 6h	0	0.00
T7M - S - 4U - 24h	0	0.00
T8M - S - 4U - CT	0	0.00
T8M - S - 4U - 1h	0.2	20.00
T8M - S - 4U - 2h	0.05	5.00
T8M - S - 4U - 3h	0.01	1.00
T8M - S - 4U - 4h	0	0.00
T8M - S - 4U - 5h	0	0.00
T8M - S - 4U - 6h	0	0.00
T8M - S - 4U - 24h	0	0.00
T9M - S - 4U - CT	0	0.00
T9M - S - 4U - 1h	0.27	27.00
T9M - S - 4U - 2h	0.17	17.00
T9M - S - 4U - 3h	0.05	5.00
T9M - S - 4U - 4h	0	0.00
T9M - S - 4U - 5h	0.01	1.00
T9M - S - 4U - 6h	0	0.00
T9M - S - 4U - 24h	0	0.00
T10M - S - 4U - CT	0	0.00
T10M - S - 4U - 1h	0.38	38.00
T10M - S - 4U - 2h	0.21	21.00
T10M - S - 4U - 3h	0.1	10.00
T10M - S - 4U - 4h	0.04	4.00
T10M - S - 4U - 5h	0.01	1.00
T10M - S - 4U - 6h	0	0.00
T10M - S - 4U - 24h	0	0.00

8.25 Serum alcohol concentrations in eugonadal males, after 8 units of alcohol

Appendix 8.25 Serum alcohol concentrations in males, after 8 units of alcohol

Male serum 8 units	Serum alcohol concentration g/L	Serum alcohol concentration mg%
T1M - S - 8U - CT	0	0
T1M - S - 8U - 1h	0.89	89
T1M - S - 8U - 2h	0.83	83
T1M - S - 8U - 3h	0.71	71
T1M - S - 8U - 4h	0.53	53
T1M - S - 8U - 5h	0.37	37
T1M - S - 8U - 6h	0.17	17
T1M - S - 8U - 24h	0	0
T2M - S - 8U - CT	0	0
T2M - S - 8U - 1h	0.83	83
T2M - S - 8U - 2h	0.74	74
T2M - S - 8U - 3h	0.55	55
T2M - S - 8U - 4h	0.36	36
T2M - S - 8U - 5h	0.16	16
T2M - S - 8U - 6h	0.04	4
T2M - S - 8U - 24h	0	0
T4M - S - 8U - CT	0	0
T4M - S - 8U - 1h	0.95	95
T4M - S - 8U - 2h	0.8	80
T4M - S - 8U - 3h	0.6	60
T4M - S - 8U - 4h	0.39	39
T4M - S - 8U - 5h	0.18	18
T4M - S - 8U - 6h	0.04	4
T4M - S - 8U - 24h	0	0
T5M - S - 8U - CT	0	0
T5M - S - 8U - 1h	0.88	88
T5M - S - 8U - 2h	0.7	70
T5M - S - 8U - 3h	0.55	55
T5M - S - 8U - 4h	0.4	40
T5M - S - 8U - 5h	0.26	26
T5M - S - 8U - 6h	0.25	25
T5M - S - 8U - 24h	0	0
T6M - S - 8U - CT	0	0
T6M - S - 8U - 1h	1.04	104
T6M - S - 8U - 2h	0.94	94
T6M - S - 8U - 3h	0.88	88
T6M - S - 8U - 4h	0.76	76
T6M - S - 8U - 5h	0.57	57
T6M - S - 8U - 6h	0.43	43
T6M - S - 8U - 24h	0	0
T7M - S - 8U - CT	0	0
T7M - S - 8U - 1h	0.86	86
T7M - S - 8U - 2h	0.71	71
T7M - S - 8U - 3h	0.53	53
T7M - S - 8U - 4h	0.38	38

T7M - S - 8U - 5h	0.19	19
T7M - S - 8U - 6h	0.05	5
T7M - S -8U - 24h	0	0
T8M - S - 8U - CT	0	0
T8M - S - 8U - 1h	0.64	64
T8M - S - 8U - 2h	0.8	80
T8M - S - 8U - 3h	0.42	42
T8M - S - 8U - 4h	0.27	27
T8M - S - 8U - 5h	0.08	8
T8M - S - 8U - 6h	0.01	1
T8M - S - 8U - 24h	0	0
T9M - S - 8U - CT	0	0
T9M - S - 8U - 1h	0.81	81
T9M - S - 8U - 2h	0.88	88
T9M - S - 8U - 3h	0.65	65
T9M - S - 8U - 4h	0.48	48
T9M - S - 8U - 5h	0.39	39
T9M - S - 8U - 6h	0.1	10
T9M - S - 8U - 24h	0	0
T10M - S - 8U-CT	0	0
T10M - S - 8U - 1h	1.11	111
T10M - S - 8U - 2h	0.78	78
T10M - S - 8U - 3h	0.78	78
T10M - S - 8U - 4h	0.56	56
T10M - S - 8U - 5h	0.37	37
T10M - S - 8U - 6h	0.15	15
T10M -S - 8U - 24h	0	0

8.26 Serum alcohol concentrations in females, after 4 units of alcohol

Appendix 8.26 Serum alcohol concentrations in females, after 4 units of alcohol

Female serum 4 units	Serum alcohol concentration g/L	Serum alcohol concentration mg%
T1F - S - 4U - CT	0	0
T1F - S - 4U - 1h	0.3	30
T1F - S - 4U - 2h	0.09	9
T1F - S - 4U - 3h	0.01	1
T1F - S - 4U - 4h	0.01	1
T1F - S - 4U - 5h	0.01	1
T1F - S - 4U - 6h	0	0
T1F - S - 4U - 24h	0	0
T2F - S - 4U - CT	0	0
T2F - S - 4U - 1h	0.51	51
T2F - S - 4U - 2h	0.34	34
T2F - S - 4U - 3h	0.19	19
T2F - S - 4U - 4h	0.04	4
T2F - S - 4U - 5h	0.01	1
T2F - S - 4U - 6h	0	0
T2F - S - 4U - 24h	0	0

T4F - S - 4U - CT	0	0
T4F - S - 4U - 1h	0.61	61
T4F - S - 4U - 2h	0.47	47
T4F - S - 4U - 3h	0.27	27
T4F - S - 4U - 4h	0.11	11
T4F - S - 4U - 5h	0.03	3
T4F - S - 4U - 6h	0.01	1
T4F - S - 4U - 24h	0	0
T5F - S - 4U - CT	0	0
T5F - S - 4U - 1h	0.53	53
T5F - S - 4U - 2h	0.35	35
T5F - S - 4U - 3h	0.18	18
T5F - S - 4U - 4h	0.06	6
T5F - S - 4U - 5h	0.01	1
T5F - S - 4U - 6h	0	0
T5F - S - 4U - 24h	0	0
T6F - S - 4U - CT	0	0
T6F - S - 4U - 1h	0.55	55
T6F - S - 4U - 2h	0.4	40
T6F - S - 4U - 3h	0.2	20
T6F - S - 4U - 4h	0.08	8
T6F - S - 4U - 5h	0.05	5
T6F - S - 4U - 6h	0.03	3
T6F - S - 4U - 24h	0	0
T7F - S - 4U - CT	0	0
T7F - S - 4U - 1h	0.53	53
T7F - S - 4U - 2h	0.38	38
T7F - S - 4U - 3h	0.15	15
T7F - S - 4U - 4h	0.02	2
T7F - S - 4U - 5h	0	0
T7F - S - 4U - 6h	0	0
T7F - S - 4U - 24h	0	0
T8F - S - 4U - CT	0	0
T8F - S - 4U - 1h	0.59	59
T8F - S - 4U - 2h	0.4	40
T8F - S - 4U - 3h	0.19	19
T8F - S - 4U - 4h	0.06	6
T8F - S - 4U - 5h	0.01	1
T8F - S - 4U - 6h	0	0
T8F - S - 4U - 24h	0	0
T9F - S - 4U - CT	0	0
T9F - S - 4U - 1h	0.48	48
T9F - S - 4U - 2h	0.32	32
T9F - S - 4U - 3h	0.14	14
T9F - S - 4U - 4h	0.03	3
T9F - S - 4U - 5h	0.01	1
T9F - S - 4U - 6h	0	0
T9F - S - 4U - 24h	0	0
T10F - S - 4U - CT	0	0
T10F - S - 4U - 1h	0.28	28
T10F - S - 4U - 2h	0.14	14
T10F - S - 4U - 3h	0.02	2
T10F - S - 4U - 4h	0.01	1

T10F - S - 4U - 5h	0	0
T10F - S - 4U - 6h	0	0
T10F - S - 4U - 24h	0	0

8.27 Serum alcohol concentrations in females, after 8 units of alcohol

Appendix 8.27 Serum alcohol concentrations in females, after 8 units of alcohol

Female serum 8 units	Serum alcohol concentration g/L	Serum alcohol concentration mg%
T1F - S - 8U - CT	0	0
T1F - S - 8U - 1h	1.02	102
T1F - S - 8U - 2h	0.9	90
T1F - S - 8U - 3h	0.64	64
T1F - S - 8U - 4h	0.29	29
T1F - S - 8U - 5h	0.1	10
T1F - S - 8U - 6h	0.02	2
T1F - S - 8U - 24h	0	0
T2F - S - 8U - CT	0	0
T2F - S - 8U - 1h	1.23	123
T2F - S - 8U - 2h	1.18	118
T2F - S - 8U - 3h	1	100
T2F - S - 8U - 4h	0.71	71
T2F - S - 8U - 5h	0.6	60
T2F - S - 8U - 6h	0.36	36
T2F - S - 8U - 24h	0	0
T4F - S - 8U - CT	0	0
T4F - S - 8U - 1h	1.08	108
T4F - S - 8U - 2h	0.92	92
T4F - S - 8U - 3h	0.75	75
T4F - S - 8U - 4h	0.61	61
T4F - S - 8U - 5h	0.39	39
T4F - S - 8U - 6h	0.23	23
T4F - S - 8U - 24h	0	0
T5F - S - 8U - CT	0	0
T5F - S - 8U - 1h	1.31	131
T5F - S - 8U - 2h	1.13	113
T5F - S - 8U - 3h	0.95	95
T5F - S - 8U - 4h	0.53	53
T5F - S -8U - 5h	0.76	76
T5F - S - 8U - 6h	0.29	29
T5F - S - 8U - 24h	0	0
T6F - S - 8U - CT	0	0
T6F - S - 8U - 1h	1.39	139
T6F - S - 8U - 2h	1.33	133
T6F - S - 8U - 3h	1.06	106
T6F - S - 8U - 4h	0.86	86
T6F - S - 8U - 5h	0.59	59
T6F - S - 8U - 6h	0.36	36
T6F - S - 8U - 24h	0	0
T7F - S - 8U - CT	0	0
T7F - S - 8U - 1h	1.27	127
T7F - S - 8U - 2h	1.12	112
T7F - S - 8U - 3h	0.88	88
T7F - S - 8U - 4h	0.63	63

T7F - S - 8U - 5h	0.41	41
T7F - S - 8U - 6h	0.24	24
T7F - S - 8U - 24h	0	0
T8F - S - 8U - CT	0	0
T8F - S - 8U - 1h	1.42	142
T8F - S - 8U - 2h	1.29	129
T8F - S - 8U - 3h	1.1	110
T8F - S - 8U - 4h	0.87	87
T8F - S - 8U - 5h	0.63	63
T8F - S - 8U - 6h	0.43	43
T8F - S - 8U - 24h	0	0
T9F - S - 8U - CT	0	0
T9F - S - 8U - 1h	1.11	111
T9F - S - 8U - 2h	1.13	113
T9F - S - 8U - 3h	0.97	97
T9F - S - 8U - 4h	0.8	80
T9F - S - 8U - 5h	0.65	65
T9F - S - 8U - 6h	0.41	41
T9F - S - 8U - 24h	0	0
T10F - S - 8U - CT	0	0
T10F - S - 8U - 1h	1.08	108
T10F - S - 8U - 2h	0.9	90
T10F - S - 8U - 3h	0.67	67
T10F - S - 8U - 4h	0.49	49
T10F - S - 8U - 5h	0.31	31
T10F - S - 8U - 6h	0.13	13
T10F - S - 8U - 24h	0	0

8.28 Internal standard mixture (working solution) in ng/mL

Appendix 8.28 Internal standard mixture (working solution) in ng/mL

Deuterated steroid	Final concentration (ng/mL)
d_3-testosterone	100
d_3-epitestosterone	4
d_3-androstenedione	20
d_3-TG	20
d_3-EG	20
d_3-TS	20
d_3-ES	20

8.29 Internal standard mixture at the concentration present in sample (ng/mL)

Appendix 8.29 Internal standard mixture at the concentration present in sample (ng/mL)

Deuterated steroid	Concentration in sample (ng/mL)
d_3-testosterone	5
d_3-epitestosterone	0.2
d_3-androstenedione	1
d_3-TG	1
d_3-EG	1
d_3-TS	1
d_3-ES	1

8.30 Serum eugonadal male concentrations of T, E, AD, TG, TS and ES (ng/mL) after 4 units (LC-MS/MS)[13]

Appendix 8.30 Serum eugonadal male concentrations after 4 units (LC-MS/MS)

Male serum 4 units	T conc (ng/mL)	E conc (ng/mL)	AD conc (ng/mL)	TG conc (ng/mL)	TS conc (ng/mL)	ES conc (ng/mL)
T1M - S - 4U - CT	5.404	0.040	0.420	-0.092	0.623	0.166
T1M - S - 4U - 1h	3.479	0.029	0.280	-0.099	0.867	0.097
T1M - S - 4U - 2h	3.840	0.025	0.382	-0.094	0.869	0.132
T1M - S - 4U - 3h	3.082	0.334	0.421	-0.099	0.790	0.072
T1M - S - 4U - 4h	3.759	0.023	0.293	-0.088	0.792	0.075
T1M - S - 4U - 5h	3.074	0.024	0.299	-0.101	0.677	0.069
T1M - S - 4U - 6h	2.842	0.019	0.243	-0.099	0.675	0.070
T1M - S - 4U - 24h	4.859	0.034	0.529	-0.087	0.707	0.181
T2M - S - 4U - CT	3.970	0.059	0.274	0.745	-0.088	0.016
T2M - S - 4U - 1h	2.930	0.394	0.182	0.746	-0.061	-0.001
T2M - S - 4U - 2h	3.212	0.049	0.259	0.707	-0.061	0.073
T2M - S - 4U - 3h	3.188	0.042	0.203	0.587	-0.027	-0.024
T2M - S - 4U - 4h	3.495	0.044	0.201	0.652	-0.077	0.013
T2M - S - 4U - 5h	3.099	0.039	0.248	0.642	-0.098	0.067
T2M - S - 4U - 6h	3.110	0.044	0.328	0.676	-0.136	0.018
T2M - S - 4U - 48h	4.146	0.076	0.464	0.653	-0.027	0.085
T4M - S - 4U - CT	1.774	0.041	0.456	0.207	-0.273	0.088
T4M - S - 4U - 1h	2.209	0.044	0.488	0.203	-0.279	-0.026

[13] The negative concentrations found in these tables were the ones below the LLOQ.

T4M - S - 4U - 2h	2.319	0.035	0.243	0.233	-0.245	0.063
T4M - S - 4U - 3h	2.430	0.103	0.434	0.178	-0.219	-0.005
T4M - S - 4U - 4h	2.454	0.184	0.433	0.203	-0.254	0.007
T4M - S - 4U - 5h	2.513	0.057	0.356	0.221	-0.310	0.016
T4M - S - 4U - 6h	2.506	0.062	0.340	0.233	-0.262	0.021
T4M - S - 4U - 24h	2.891	0.082	0.362	0.277	-0.247	0.069
T5M - S - 4U - CT	5.688	0.067	0.694	0.071	4.112	0.359
T5M - S - 4U - 1h	5.068	0.023	0.422	-0.079	6.146	0.381
T5M - S - 4U - 2h	5.054	0.032	0.622	-0.074	7.075	0.385
T5M - S - 4U - 3h	5.353	0.039	0.554	-0.083	6.226	0.367
T5M - S - 4U - 4h	4.787	0.027	0.555	-0.087	4.812	0.328
T5M - S - 4U - 5h	4.702	0.061	0.516	0.050	4.852	0.347
T5M - S - 4U - 6h	4.803	0.032	0.485	-0.072	4.831	0.349
T5M - S - 4U - 24h	6.273	0.033	0.717	-0.081	4.826	0.408
T6M - S - 4U -CT	3.475	0.051	0.786	-0.095	0.227	0.206
T6M - S - 4U - 1h	3.475	0.040	0.310	-0.100	0.463	0.164
T6M - S - 4U - 2h	4.065	0.043	0.350	-0.104	0.639	0.208
T6M - S - 4U - 3h	3.009	0.041	0.535	-0.103	0.658	0.198
T6M - S - 4U - 4h	3.274	0.041	0.723	-0.099	0.611	0.166
T6M - S - 4U - 5h	2.590	0.042	0.406	-0.106	0.563	0.161
T6M - S - 4U - 6h	2.557	0.040	0.340	-0.099	0.459	0.151
T6M - S - 4U - 24h	2.496	0.044	0.681	-0.099	0.190	0.183
T7M - S - 4U - CT	4.290	0.056	0.456	0.590	0.549	0.222
T7M - S - 4U - 1h	3.114	0.036	0.383	0.584	0.678	0.167
T7M - S - 4U - 2h	3.202	0.049	0.476	0.578	0.718	0.225
T7M - S - 4U - 3h	2.763	0.061	0.383	0.427	0.523	0.145
T7M - S - 4U - 4h	3.364	0.051	0.568	0.407	0.644	0.239
T7M - S - 4U - 5h	3.361	0.053	0.508	0.409	0.555	0.287
T7M - S - 4U - 6h	3.339	0.049	0.526	0.320	0.506	0.237
T7M - S - 4U - 24h	3.747	0.071	0.683	0.427	0.409	0.179
T8M - S - 4U - CT	3.724	0.044	0.723	0.707	1.791	0.382
T8M - S - 4U - 1h	3.497	0.043	0.396	0.928	2.638	0.361
T8M - S - 4U - 2h	3.054	0.031	0.651	0.710	2.888	0.299
T8M - S - 4U - 3h	2.926	0.031	0.642	0.503	2.381	0.288
T8M - S - 4U - 4h	2.549	0.034	0.729	0.563	2.363	0.415
T8M - S - 4U - 5h	2.853	0.041	0.886	0.720	2.162	0.473
T8M - S - 4U - 6h	3.491	0.045	0.546	0.652	2.066	0.454
T8M - S - 4U - 24h	3.929	0.082	0.980	0.561	1.494	0.442
T9M - S - 4U - CT	2.945	0.048	0.540	0.441	0.056	0.227
T9M - S - 4U - 1h	2.442	0.042	0.383	0.622	0.184	0.144
T9M - S - 4U - 2h	2.397	0.041	0.443	0.445	0.204	0.153
T9M - S - 4U - 3h	3.126	0.044	0.598	0.478	0.358	0.228
T9M - S - 4U - 4h	2.646	0.051	0.686	0.443	0.211	0.253
T9M - S - 4U - 5h	2.379	0.037	0.728	0.274	0.162	0.252
T9M - S - 4U - 6h	1.622	0.038	0.591	0.251	0.086	0.185
T9M - S - 4U - 24h	2.424	0.056	0.751	0.501	0.012	0.158
T10M - S - 4U - CT	4.889	0.062	0.551	-0.072	0.979	0.302
T10M - S - 4U - 1h	3.319	0.028	0.219	-0.107	2.117	0.150
T10M - S - 4U - 2h	4.400	0.028	0.240	-0.097	2.609	0.174
T10M - S - 4U - 3h	4.218	0.034	0.240	-0.081	2.470	0.145
T10M - S - 4U - 4h	4.530	0.037	0.278	-0.086	1.748	0.140
T10M - S - 4U - 5h	5.760	0.035	0.451	-0.088	1.467	0.334
T10M - S - 4U - 6h	5.140	0.035	0.350	-0.104	1.493	0.191

T10M - S - 4U - 24h	5.126	0.074	0.504	-0.046	0.979	0.238

8.31 Serum female concentrations of T, E, AD, TG, TS and ES (ng/mL) after 4 units (LC-MS/MS)

Appendix 8.31 Serum female concentrations after 4 units (LC-MS/MS)

Female serum 4 units	T conc (ng/mL)	E conc (ng/mL)	AD conc (ng/mL)	TG conc (ng/mL)	TS conc (ng/mL)	ES conc (ng/mL)
T1F - S - 4U - CT	0.300	0.028	1.330	-0.029	0.285	-0.014
T1F - S - 4U - 1h	0.314	0.024	0.739	0.019	0.233	-0.071
T1F - S - 4U - 2h	0.311	0.034	0.968	0.009	0.217	-0.071
T1F - S - 4U - 3h	0.300	0.026	1.146	-0.024	0.253	-0.015
T1F - S - 4U - 4h	0.339	0.030	0.961	-0.014	0.237	-0.062
T1F - S - 4U - 5h	0.302	0.027	0.946	-0.039	0.242	-0.056
T1F - S - 4U - 6h	0.282	0.026	1.063	-0.044	0.227	-0.040
T1F - S - 4U - 24h	0.271	0.032	1.481	-0.059	0.122	-0.042
T2F - S - 4U - CT	0.216	0.029	1.456	0.039	-0.106	0.001
T2F - S - 4U - 1h	0.275	0.018	0.753	0.099	-0.155	-0.069
T2F - S - 4U - 2h	0.323	0.020	1.096	0.127	-0.078	0.011
T2F - S - 4U - 3h	0.279	0.020	1.025	0.203	-0.048	-0.049
T2F - S - 4U - 4h	0.236	0.016	1.056	0.031	-0.017	-0.031
T2F - S - 4U - 5h	0.254	0.020	1.137	-0.001	-0.008	-0.040
T2F - S - 4U - 6h	0.212	0.021	1.100	0.007	-0.024	-0.026
T2F - S - 4U - 24h	0.210	0.021	1.600	0.013	-0.096	0.107
T4F - S - 4U - CT	0.243	0.037	1.024	0.053	-0.155	0.000
T4F - S - 4U - 1h	0.267	0.028	0.902	0.085	-0.179	-0.024
T4F - S - 4U - 2h	0.282	0.035	0.965	0.225	-0.193	0.090
T4F - S - 4U - 3h	0.348	0.035	1.120	0.197	-0.159	0.047
T4F - S - 4U - 4h	0.330	0.039	1.079	0.189	-0.159	-0.013
T4F - S - 4U - 5h	0.246	0.031	0.909	0.077	-0.148	-0.053
T4F - S - 4U - 6h	0.227	0.028	0.908	0.002	-0.074	-0.038
T4F - S - 4U - 24h	0.282	0.050	1.267	-0.033	-0.172	0.064
T5F - S - 4U - CT	0.415	0.047	1.640	0.104	-0.201	-0.037
T5F - S - 4U - 1h	0.611	0.046	0.992	0.386	0.048	0.021
T5F - S - 4U - 2h	0.606	0.041	0.848	0.447	0.157	0.004
T5F - S - 4U - 3h	0.518	0.039	0.865	0.384	0.098	-0.042
T5F - S - 4U - 4h	0.355	0.042	1.190	0.289	0.148	-0.027
T5F - S - 4U - 5h	0.316	0.043	1.130	0.144	0.032	0.007
T5F - S - 4U - 6h	0.343	0.037	1.277	0.090	-0.042	-0.033
T5F - S - 4U - 24h	0.432	0.048	1.629	0.035	-0.151	-0.028
T6F - S - 4U - CT	0.224	0.031	1.176	0.042	0.419	-0.056
T6F - S - 4U - 1h	0.253	0.031	0.972	0.160	0.043	-0.018
T6F - S - 4U - 2h	0.229	0.022	1.089	0.152	0.051	-0.070
T6F - S - 4U - 3h	0.300	0.028	1.632	0.235	0.084	0.040
T6F - S - 4U - 4h	0.295	0.058	1.762	0.081	0.390	0.008
T6F - S - 4U - 5h	0.327	0.066	1.307	0.038	0.474	-0.026
T6F - S - 4U - 6h	0.265	0.035	1.151	0.051	0.316	-0.070
T6F - S - 4U - 24h	0.201	0.032	1.828	0.067	0.219	0.054
T7F - S - 4U - CT	0.448	0.025	1.386	0.061	-0.340	-0.015
T7F - S - 4U - 1h	0.546	0.009	0.935	0.195	-0.310	-0.058

T7F - S - 4U - 2h	0.505	0.007	0.961	0.157	-0.308	-0.055
T7F - S - 4U - 3h	0.465	0.016	1.037	0.179	-0.356	-0.060
T7F - S - 4U - 4h	0.410	0.014	1.148	0.092	-0.331	-0.054
T7F - S - 4U - 5h	0.390	0.014	1.057	0.059	-0.346	-0.091
T7F - S - 4U - 6h	0.375	0.011	1.051	0.025	-0.342	-0.092
T7F - S - 4U - 24h	0.384	0.024	1.275	0.069	-0.360	0.024
T8F - S - 4U - CT	0.267	0.011	1.016	-0.017	-0.258	-0.065
T8F - S - 4U - 1h	0.348	0.006	0.747	0.054	-0.254	-0.096
T8F - S - 4U - 2h	0.419	0.007	0.845	0.157	-0.238	-0.036
T8F - S - 4U - 3h	0.432	0.011	0.917	0.123	-0.215	-0.047
T8F - S - 4U - 4h	0.324	0.025	1.058	0.063	-0.233	-0.042
T8F - S - 4U - 5h	0.293	0.009	1.176	0.035	-0.219	-0.059
T8F - S - 4U - 6h	0.254	0.008	1.028	0.068	-0.250	-0.069
T8F - S - 4U - 24h	0.223	0.015	1.060	0.009	-0.288	0.033
T9F - S - 4U - CT	0.212	-0.004	0.803	-0.018	-0.174	-0.040
T9F - S - 4U - 1h	0.284	-0.005	0.533	0.216	-0.228	-0.020
T9F - S - 4U - 2h	0.238	-0.004	0.498	0.162	-0.242	-0.111
T9F - S - 4U - 3h	0.255	-0.007	0.668	0.207	-0.196	-0.075
T9F - S - 4U - 4h	0.231	0.003	0.975	0.136	-0.136	-0.008
T9F - S - 4U - 5h	0.257	-0.002	0.848	0.070	-0.165	-0.056
T9F - S - 4U - 6h	0.217	-0.006	0.846	0.020	-0.140	-0.068
T9F - S - 4U - 24h	0.201	-0.001	0.945	0.013	-0.171	-0.052
T10F - S - 4U - CT	0.122	-0.004	0.939	0.060	-0.362	-0.025
T10F - S - 4U - 1h	0.207	-0.001	0.640	0.226	-0.273	-0.093
T10F - S - 4U - 2h	0.217	-0.003	0.788	0.209	-0.252	-0.091
T10F - S - 4U - 3h	0.248	-0.003	1.065	0.218	-0.283	0.020
T10F - S - 4U - 4h	0.252	0.006	1.124	0.157	-0.290	-0.029
T10F - S - 4U - 5h	0.282	0.004	1.158	0.184	-0.307	0.007
T10F - S - 4U - 6h	0.274	-0.003	1.122	0.176	-0.312	-0.054
T10F - S - 4U - 24h	0.239	-0.004	1.594	0.170	-0.295	-0.022

8.32 Serum eugonadal male concentrations of T, E, AD, TG, TS and ES (ng/mL) after 8 units (LC-MS/MS)

Appendix 8.32 Serum male concentrations after 8 units (LC-MS/MS)

Male serum 8 units	T conc (ng/mL)	E conc (ng/mL)	AD conc (ng/mL)	TG conc (ng/mL)	TS conc (ng/mL)	ES conc (ng/mL)
T1M - S - 8U - CT	4.937	0.084	0.335	0.024	0.792	0.028
T1M - S - 8U - 1h	4.198	0.039	0.205	-0.008	1.142	0.085
T1M - S - 8U - 2h	3.865	0.035	0.190	0.034	1.340	0.051
T1M - S - 8U - 3h	4.905	0.041	0.272	-0.029	1.558	0.096
T1M - S - 8U - 4h	5.038	0.045	0.285	-0.003	1.414	0.062
T1M - S - 8U - 5h	4.469	0.032	0.272	-0.034	1.621	0.045
T1M - S - 8U - 6h	2.970	0.030	0.191	-0.048	1.306	-0.022
T1M - S - 8U - 24h	4.472	0.046	0.393	-0.057	1.218	0.077
T1M - S - 4U - 48h	5.275	0.049	0.474	-0.039	1.355	0.178
T2M - S - 8U - CT	4.114	0.085	1.303	0.559	0.071	-0.096
T2M - S - 8U - 1h	3.024	0.043	0.493	0.747	0.217	-0.102
T2M - S - 8U - 2h	3.407	0.045	0.197	0.879	0.252	-0.134
T2M - S - 8U - 3h	3.638	0.056	0.189	0.878	0.202	-0.133

T2M - S - 8U - 4h	3.721	0.056	0.202	0.919	0.208	-0.116
T2M - S - 8U - 5h	3.638	0.055	0.286	0.773	0.193	-0.071
T2M - S - 8U - 6h	3.501	0.058	0.534	0.543	0.221	-0.103
T2M - S - 4U - 24h	4.541	0.186	0.496	0.560	0.145	-0.081
T2M - S - 4U - 48h	3.213	0.089	0.699	0.758	0.110	0.110
T4M - S - 8U - CT	2.228	0.063	0.603	0.251	-0.164	0.257
T4M - S - 8U - 1h	1.720	0.048	0.760	0.293	-0.121	0.099
T4M - S - 8U - 2h	1.871	0.050	0.463	0.365	-0.120	0.037
T4M - S - 8U - 3h	2.072	0.057	0.322	0.372	-0.154	0.101
T4M - S - 8U - 4h	2.386	0.062	0.378	0.431	-0.096	0.137
T4M - S - 8U - 5h	2.338	0.061	0.538	0.394	-0.105	0.152
T4M - S - 8U - 6h	2.285	0.059	0.493	0.316	-0.089	0.125
T4M - S - 8U - 24h	2.205	0.063	0.405	0.276	-0.125	0.257
T4M - S - 8U - 48h	2.218	0.062	0.646	0.299	-0.125	0.261
T5M - S - 8U - CT	5.574	0.029	0.660	-0.040	3.352	0.542
T5M - S - 8U - 1h	2.542	0.015	1.135	-0.037	4.364	0.233
T5M - S - 8U - 2h	2.046	0.011	0.286	-0.041	4.431	0.289
T5M - S - 8U - 3h	1.796	0.016	0.846	-0.039	4.285	0.151
T5M - S - 8U - 4h	3.403	0.018	0.407	-0.042	4.256	0.191
T5M - S - 8U - 5h	3.029	0.014	0.460	-0.039	4.644	0.235
T5M - S - 8U - 6h	3.199	0.043	0.419	0.740	0.397	0.266
T5M - S - 4U - 24h	3.852	0.019	0.692	-0.035	3.888	0.344
T5M - S - 8U - 48h	3.766	0.021	0.765	-0.038	3.855	0.384
T6M - S - 8U - CT	3.609	0.061	0.736	-0.032	0.306	0.238
T6M - S - 8U - 1h	3.595	0.054	0.831	-0.043	0.537	0.242
T6M - S - 8U - 2h	3.462	0.052	0.539	-0.045	0.649	0.194
T6M - S - 8U - 3h	4.015	0.050	0.404	-0.040	0.729	0.201
T6M - S - 8U - 4h	3.442	0.058	0.499	-0.036	0.806	0.192
T6M - S - 8U - 5h	3.324	0.060	0.459	-0.034	0.783	0.179
T6M - S - 8U - 6h	2.970	0.046	0.443	0.004	0.689	0.171
T6M - S - 8U - 24h	3.481	0.052	0.397	-0.035	0.449	0.191
T6M - S - 8U - 48h	4.231	0.069	0.624	-0.015	0.599	0.266
T7M - S - 8U - CT	3.164	0.064	0.901	0.506	0.391	0.191
T7M - S - 8U - 1h	2.452	0.042	0.502	0.530	0.631	0.156
T7M - S - 8U - 2h	2.188	0.055	0.356	0.492	0.627	0.180
T7M - S - 8U - 3h	2.874	0.059	0.414	0.576	0.610	0.139
T7M - S - 8U - 4h	3.288	0.069	0.405	0.711	0.647	0.214
T7M - S - 8U - 5h	3.056	0.071	0.402	0.731	0.655	0.266
T7M - S - 8U - 6h	2.855	0.058	0.599	0.599	0.644	0.198
T7M - S -8U - 24h	2.943	0.049	0.619	0.326	0.333	0.252
T7M - S -8U - 48h	3.597	0.063	0.663	0.275	0.380	0.250
T8M - S - 8U - CT	5.005	0.066	0.837	0.603	2.015	0.663
T8M - S - 8U - 1h	4.069	0.068	0.782	1.088	3.202	0.477
T8M - S - 8U - 2h	3.840	0.072	0.378	1.105	2.999	0.453
T8M - S - 8U - 3h	5.384	0.051	0.492	1.300	4.134	0.440
T8M - S - 8U - 4h	5.179	0.043	0.525	1.430	4.376	0.411
T8M - S - 8U - 5h	5.040	0.045	0.636	1.248	4.867	0.484
T8M - S - 8U - 6h	4.249	0.033	0.828	1.182	4.969	0.536
T8M - S - 8U - 24h	4.967	0.055	0.970	0.504	2.319	0.475
T8M - S - 8U - 48h	3.585	0.036	0.716	0.885	2.172	0.467
T9M - S - 8U - CT	2.187	0.032	0.538	0.475	0.101	0.185
T9M - S - 8U - 1h	2.163	0.029	0.239	0.450	0.176	0.102
T9M - S - 8U - 2h	2.942	0.016	0.168	-0.051	1.069	0.088

T9M - S - 8U - 3h	2.435	0.027	0.340	0.545	0.205	0.130
T9M - S - 8U - 4h	2.897	0.034	0.474	0.666	0.322	0.151
T9M - S - 8U - 5h	2.683	0.029	0.598	0.613	0.269	0.164
T9M - S - 8U - 6h	3.561	0.010	0.709	-0.051	5.514	0.259
T9M - S - 8U - 24h	2.161	0.023	0.558	0.441	0.159	0.186
T9M - S - 8U - 48h	2.406	0.026	0.741	0.492	0.203	0.224
T10M - S - 8U-CT	3.09	0.02	0.28	-0.03	0.85	0.15
T10M - S - 8U - 1h	2.73	0.01	0.18	-0.04	1.25	0.13
T10M - S - 8U - 2h	3.05	0.04	0.24	0.54	0.25	0.11
T10M - S - 8U - 3h	3.44	0.02	0.15	-0.08	1.29	0.08
T10M - S - 8U - 4h	3.58	0.03	0.21	-0.08	1.47	0.09
T10M - S - 8U - 5h	3.49	0.03	0.21	-0.10	1.40	0.12
T10M - S - 8U - 6h	3.45	0.03	0.22	-0.08	1.47	0.12
T10M - S - 8U-24h	3.71	0.03	0.39	-0.10	1.04	0.17
T10M - S - 8U-48h	3.90	0.03	0.38	-0.07	0.90	0.15

8.33 Serum female concentrations of T, E, AD, TG, TS and ES (ng/mL) after 8 units (LC-MS/MS)

Appendix 8.33 Serum female concentrations after 8 units (LC-MS/MS)

Female serum 8 units	T conc (ng/mL)	E conc (ng/mL)	AD conc (ng/mL)	TG conc (ng/mL)	TS conc (ng/mL)	ES conc (ng/mL)
T1F - S - 8U - CT	0.208	0.027	0.753	-0.027	0.133	-0.038
T1F - S - 8U - 1h	0.096	0.010	0.197	-0.033	-0.102	-0.050
T1F - S - 8U - 2h	0.252	0.009	0.538	0.094	0.104	-0.040
T1F - S - 8U - 3h	0.250	0.010	0.507	0.081	0.067	-0.030
T1F - S - 8U - 4h	0.320	0.012	0.746	0.138	0.098	-0.029
T1F - S - 8U - 5h	0.264	0.015	0.812	0.092	0.090	-0.018
T1F - S - 8U - 6h	0.258	0.011	0.691	0.014	0.239	-0.020
T1F - S - 8U - 24h	0.226	0.015	1.125	-0.013	0.114	0.013
T1F - S - 8U - 48h	0.234	0.014	1.189	-0.001	-0.012	0.014
T2F - S - 8U - CT	0.226	0.013	0.699	0.159	0.035	-0.037
T2F - S - 8U - 1h	0.254	0.025	1.521	0.157	0.005	0.041
T2F - S - 8U - 2h	0.244	0.015	0.892	0.225	0.012	-0.016
T2F - S - 8U - 3h	0.225	0.011	0.687	0.158	0.012	-0.041
T2F - S - 8U - 4h	0.252	0.013	0.829	0.205	-0.023	-0.041
T2F - S - 8U - 5h	0.272	0.012	0.904	0.244	0.013	-0.020
T2F - S - 8U - 6h	0.288	0.021	1.027	0.343	0.076	0.032
T2F - S - 8U - 24h	0.276	0.018	1.892	0.035	0.011	0.036
T2F - S - 8U - 48h	0.235	0.020	1.299	0.028	-0.001	-0.016
T4F - S - 8U - CT	0.157	0.015	0.637	-0.022	-0.160	0.008
T4F - S - 8U - 1h	0.153	0.014	0.278	0.005	-0.191	-0.046
T4F - S - 8U - 2h	0.146	0.010	0.219	0.001	-0.216	-0.050
T4F - S - 8U - 3h	0.133	0.010	0.187	-0.006	-0.208	-0.052
T4F - S - 8U - 4h	0.140	0.011	0.156	-0.005	-0.220	-0.040
T4F - S - 8U - 5h	0.172	0.012	0.264	0.020	-0.175	-0.037
T4F - S - 8U - 6h	0.186	0.013	0.378	0.060	-0.176	-0.023
T4F - S - 8U - 24h	0.129	0.012	0.445	-0.026	-0.111	-0.015
T4F - S - 8U - 48h	0.119	0.010	0.444	-0.051	-0.166	-0.029
T5F - S - 8U - CT	0.251	0.023	1.022	0.009	-0.122	-0.037

T5F - S - 8U - 1h	0.363	0.018	0.381	0.277	0.072	-0.017
T5F - S - 8U - 2h	0.386	0.021	0.302	0.116	0.086	0.038
T5F - S - 8U - 3h	0.395	0.011	0.295	0.129	0.160	0.042
T5F - S - 8U - 4h	0.346	0.015	0.247	0.134	0.205	0.050
T5F - S -8U - 5h	0.395	0.017	0.310	0.164	0.198	0.118
T5F - S - 8U - 6h	0.405	0.021	0.354	0.223	0.233	0.078
T5F - S - 8U - 24h	0.165	0.019	0.898	0.029	0.134	0.099
T5F - S - 8U - 48h	0.163	0.017	0.912	0.047	0.218	0.107
T6F - S - 8U - CT	0.284	0.018	1.749	0.018	0.519	0.117
T6F - S - 8U - 1h	0.205	0.031	0.856	0.128	0.208	0.107
T6F - S - 8U - 2h	0.194	0.013	0.862	0.176	0.158	0.147
T6F - S - 8U - 3h	0.216	0.107	0.876	0.143	0.252	0.112
T6F - S - 8U - 4h	0.209	0.024	1.027	0.184	0.260	0.102
T6F - S - 8U - 5h	0.300	0.037	1.310	0.263	0.319	0.138
T6F - S - 8U - 6h	0.380	0.301	1.529	0.318	0.324	0.138
T6F - S - 8U - 24h	0.237	0.032	2.043	0.054	0.695	0.196
T6F - S - 8U - 48h	0.231	0.025	1.933	0.075	0.575	0.226
T7F - S - 8U - CT	0.520	0.054	1.943	0.048	-0.267	0.112
T7F - S - 8U - 1h	0.588	0.038	1.131	0.142	-0.300	0.095
T7F - S - 8U - 2h	0.643	0.036	1.337	0.128	-0.321	0.167
T7F - S - 8U - 3h	0.538	0.030	0.992	0.105	-0.302	0.086
T7F - S - 8U - 4h	0.653	0.038	1.304	0.173	-0.289	0.137
T7F - S - 8U - 5h	0.653	0.041	1.264	0.206	-0.274	0.109
T7F - S - 8U - 6h	0.653	0.036	1.300	0.243	-0.268	0.075
T7F - S - 8U - 24h	0.395	0.047	1.526	0.014	-0.310	0.065
T7F - S - 8U - 48h	0.407	0.073	1.753	-0.013	-0.309	0.042
T8F - S - 8U - CT	0.185	0.026	1.036	-0.038	-0.255	0.101
T8F - S - 8U - 1h	0.262	0.019	0.582	-0.032	-0.195	0.052
T8F - S - 8U - 2h	0.321	0.021	0.718	0.034	-0.195	0.026
T8F - S - 8U - 3h	0.339	0.024	0.639	0.045	-0.176	0.039
T8F - S - 8U - 4h	0.343	0.015	0.614	0.078	-0.202	-0.037
T8F - S - 8U - 5h	0.348	0.015	0.646	0.118	-0.178	-0.045
T8F - S - 8U - 6h	0.394	0.788	0.708	0.171	-0.168	-0.038
T8F - S - 8U - 24h	0.117	0.006	0.627	-0.005	-0.275	-0.038
T8F - S - 8U - 48h	0.155	0.012	0.794	-0.034	-0.261	-0.018
T9F - S - 8U - CT	0.232	0.007	1.400	0.008	-0.232	0.061
T9F - S - 8U - 1h	0.165	0.007	0.544	0.080	-0.243	-0.048
T9F - S - 8U - 2h	0.159	0.005	0.515	0.107	-0.229	-0.079
T9F - S - 8U - 3h	0.159	0.002	0.497	0.076	-0.256	-0.080
T9F - S - 8U - 4h	0.192	0.002	0.755	0.124	-0.264	-0.031
T9F - S - 8U - 5h	0.197	0.002	0.585	0.159	-0.248	-0.061
T9F - S - 8U - 6h	0.220	0.003	0.663	0.223	-0.238	-0.050
T9F - S - 8U - 24h	0.154	0.006	0.692	-0.026	-0.220	-0.055
T9F - S - 8U - 48h	0.181	0.116	1.000	0.041	-0.250	-0.049
T10F - S - 8U - CT	0.140	0.012	1.091	0.046	-0.353	-0.081
T10F - S - 8U - 1h	0.115	0.004	0.697	0.141	-0.303	-0.050
T10F - S - 8U - 2h	0.175	0.007	0.859	0.208	-0.276	-0.034
T10F - S - 8U - 3h	0.194	0.012	0.938	0.211	-0.275	-0.014
T10F - S - 8U - 4h	0.234	0.005	1.177	0.388	-0.233	0.060
T10F - S - 8U - 5h	0.230	0.007	0.831	0.253	-0.222	-0.018
T10F - S - 8U - 6h	0.233	0.008	0.913	0.268	-0.200	-0.006
T10F - S - 8U - 24h	0.188	0.170	1.719	0.051	-0.320	0.015
T10F - S - 8U - 48h	0.155	0.012	1.460	0.109	-0.334	0.051

8.34 Urinary T/E, and urinary concentrations of T, E and 5α-DHT (ng/mL) for hypogonadal men in their steady state of hormone replacement therapy

Appendix 8.34 Urinary T/E, and urinary concentrations of T, E and 5α-DHT for hypogonadal men in their steady state of hormone replacement therapy

Sample	T/E	T conc	E conc	5α-DHT conc	SG
T11M-U-CT	4.88	34.09	7.43	7.53	1.012
T11M-U-1h	5.64	26.71	4.67	4.74	1.009
T11M-U-2h	7.85	49.53	7.33	6.46	1.014
T11M-U-3h	7.05	50.18	8.26	6.74	1.016
T11M-U-4h	6.82	60.98	9.54	8.53	1.021
T11M-U-5h	6.63	57.37	10.16	9.80	1.021
T11M-U-6h	5.29	51.27	11.06	12.64	1.021
T11M-U-7h	4.73	52.18	12.12	12.62	1.019
T11M-U-10h	7.48	70.78	11.36	14.57	1.022
T11M-U-24h	5.92	28.70	5.44	7.77	1.015
T12M-U-CT	10.55	28.26	0.50	7.84	1.006
T12M-U-1h	10.07	24.83	0.50	4.74	1.007
T12M-U-2h	26.92	48.64	1.89	6.57	1.014
T12M-U-3h	29.21	72.85	2.65	8.63	1.017
T12M-U-4h	27.28	85.16	3.39	11.23	1.017
T12M-U-5h	25.23	83.11	3.74	13.57	1.017
T12M-U-6h	22.82	96.86	4.88	18.06	1.021
T12M-U-7h	18.77	66.65	3.82	17.89	1.016
T12M-U-10h	20.25	51.72	2.40	10.07	1.010
T12M -U-24h	9.50	45.26	5.22	10.07	1.007
T13M-U-CT	10.18	58.54	6.73	9.88	1.026
T13M-U-1h	10.34	27.49	0.50	4.57	1.007
T13M-U-2h	21.00	42.69	2.42	4.97	1.019
T13M-U-3h	25.05	79.56	3.33	7.86	1.027
T13M-U-4h	27.25	95.00	3.95	9.66	1.029
T13M-U-5h	24.12	111.99	5.16	12.19	1.030
T13M-U-6h	23.23	119.84	6.03	15.98	1.030
T13M-U-7h	20.50	105.44	5.86	16.40	1.028
T13M-U-10h	21.05	126.80	7.08	15.01	1.031
T13M -U-24h	12.00	27.95	2.95	5.53	1.020

8.35 Urinary T/E, and urinary concentrations of T, E and 5α-DHT for hypogonadal men in their supraphysiological phase of hormone replacement therapy

Appendix 8.35 Urinary T/E, and urinary concentrations of T, E and 5α-DHT for hypogonadal men in their supraphysiological phase of hormone replacement therapy

Sample	T/E	T conc	E conc	5α-DHT conc	SG
T16M-U-CT	20.60	125.18	9.65	21.72	1.024
T16M-U-1h	28.44	120.29	0.50	12.65	1.004
T16M-U-2h	52.75	15.98	3.16	13.28	1.009
T16M-U-3h	50.86	168.18	4.30	15.07	1.016
T16M-U-4h	55.01	227.41	5.74	19.92	1.021
T16M-U-5h	65.07	272.10	5.10	21.16	1.022
T16M-U-6h	64.48	267.93	6.03	26.05	1.022
T16M-U-7h	53.24	342.30	7.45	41.55	1.023
T16M-U-10h	40.78	294.38	9.83	39.05	1.024
T16M -U-24h	18.72	167.48	13.63	31.39	1.027
T17M-U-CT	11.71	157.85	15.74	19.60	1.012
T17M-U-1h	11.04	95.83	9.49	48.40	1.006
T17M-U-2h	13.50	138.35	12.37	16.22	1.010
T17M-U-3h	15.22	153.21	12.45	14.82	1.014
T17M-U-4h	12.61	123.93	12.74	15.28	1.019
T17M-U-5h	13.48	153.95	13.34	18.40	1.019
T17M-U-6h	12.75	146.52	15.62	25.90	1.020
T17M-U-7h	12.12	155.40	16.04	25.67	1.019
T17M-U-10h	12.97	136.64	13.23	22.07	1.019
T17M -U-24h	10.23	137.00	16.44	21.77	1.013
T18M-U-CT	16.65	95.31	6.36	16.39	1.007
T18M-U-1h	18.90	75.31	4.40	11.44	1.006
T18M-U-2h	26.43	88.28	3.70	9.06	1.014
T18M-U-3h	31.78	124.35	4.34	11.12	1.018
T18M-U-4h	35.68	124.91	4.11	11.11	1.016
T18M-U-5h	38.22	158.60	4.63	13.73	1.016
T18M-U-6h	33.72	115.49	4.43	13.32	1.016
T18M-U-7h	35.03	120.75	3.93	18.40	1.012
T18M-U-10h	36.72	158.40	5.28	22.89	1.010
T18M -U-24h	15.94	112.28	7.18	17.93	1.008

8.36 Serum concentration of T, E, AD, TG, TS and ES (ng/mL) for hypogonadal men in their steady state of hormone replacement therapy

Appendix 8.36 Serum concentration of T, E, AD, TG, TS and ES for hypogonadal men in their steady state of hormone replacement therapy

Volunteer code	T conc (ng/mL)	E conc (ng/mL)	AD conc (ng/mL)	TG conc (ng/mL)	TS conc (ng/mL)	ES conc (ng/mL)
T11M - S - 8U - CT	5.46	0.01	2.17	0.89	-0.19	0.16
T11M - S - 8U - 1h	4.41	-0.01	0.76	0.87	-0.27	-0.04
T11M - S - 8U - 2h	5.33	0.00	0.87	1.08	-0.25	-0.04
T11M - S - 8U - 3h	4.99	-0.01	1.06	1.08	-0.25	0.01
T11M - S - 8U - 4h	5.99	-0.01	1.03	1.03	-0.24	-0.07
T11M - S - 8U - 5h	5.68	-0.01	0.95	0.92	-0.18	-0.06
T11M - S - 8U - 6h	5.82	0.00	1.20	0.84	-0.22	0.04
T11M - S - 8U - 24h	8.01	0.00	0.83	0.82	-0.21	-0.06
T12M - S - 8U - CT	8.31	-0.01	0.82	0.71	-0.11	-0.09
T12M - S - 8U - 1h	7.74	-0.01	0.46	0.84	-0.08	-0.09
T12M - S - 8U - 2h	8.46	-0.01	0.77	1.02	-0.05	-0.10
T12M - S - 8U - 3h	11.16	0.00	0.98	1.01	-0.03	-0.08
T12M - S - 8U - 4h	10.42	-0.01	0.76	1.10	-0.04	-0.08
T12M - S - 8U - 5h	7.75	0.01	0.62	0.82	-0.12	0.00
T12M - S - 8U - 6h	7.80	0.00	0.59	0.90	-0.13	0.02
T12M - S - 8U - 24h	5.82	0.00	0.51	0.65	-0.18	0.04
T13M - S - 8U - CT	3.19	0.01	0.82	0.37	-0.27	0.03
T13M - S - 8U - 1h	2.02	0.00	0.30	0.57	-0.28	-0.01
T13M - S - 8U - 2h	2.53	0.00	0.24	0.86	-0.26	-0.01
T13M - S - 8U - 3h	2.79	0.00	0.26	0.95	-0.25	0.04
T13M - S - 8U - 4h	3.82	0.00	0.39	0.99	-0.25	0.00
T13M - S - 8U - 5h	3.29	0.00	0.41	1.08	-0.22	0.00
T13M - S - 8U - 6h	2.96	0.00	0.42	0.89	-0.24	0.00
T13M - S - 8U - 24h	3.81	0.00	0.48	0.78	-0.28	-0.01

8.37 Serum concentration of T, E, AD, TG, TS and ES (ng/mL) for hypogonadal men in their supraphysiological phase of hormone replacement therapy

Appendix 8.37 Serum concentration of T, E, AD, TG, TS and ES for hypogonadal men in their supraphysiological phase of hormone replacement therapy

Volunteer code	T conc (ng/mL)	E conc (ng/mL)	AD conc (ng/mL)	TG conc (ng/mL)	TS conc (ng/mL)	ES conc (ng/mL)
T16M - S - 8U - CT	9.07	0.00	0.91	1.71	-0.13	0.06
T16M - S - 8U - 1h	7.88	0.01	0.59	1.97	-0.10	0.05
T16M - S - 8U - 2h	8.28	0.00	0.54	2.07	-0.10	0.00
T16M - S - 8U - 3h	9.58	0.00	0.71	2.44	-0.08	0.05
T16M - S - 8U - 4h	10.09	0.00	0.63	2.62	-0.08	0.03
T16M - S - 8U - 5h	10.11	0.00	0.65	2.70	-0.10	0.02
T16M - S - 8U - 6h	10.68	0.01	0.63	2.70	-0.09	0.04
T16M - S - 8U - 24h	8.04	0.00	0.74	2.10	-0.14	0.03
T17M - S - 8U - CT	7.47	0.01	1.17	1.30	-0.21	0.11
T17M - S - 8U - 1h	7.04	0.00	0.69	1.55	-0.19	0.01
T17M - S - 8U - 2h	8.43	0.00	1.24	1.62	-0.19	0.03
T17M - S - 8U - 3h	8.16	0.00	0.86	1.72	-0.19	0.07
T17M - S - 8U - 4h	9.12	0.08	0.92	1.64	-0.18	0.03
T17M - S - 8U - 5h	9.48	0.01	0.89	1.81	-0.18	0.03
T17M - S - 8U - 6h	8.68	0.00	1.02	1.46	-0.18	0.07
T17M - S - 8U - 24h	9.65	0.00	0.88	1.86	-0.18	-0.01
T18M - S - 8U - CT	8.45	0.08	1.03	0.91	-0.17	0.11
T18M - S - 8U - 1h	5.27	0.00	0.26	1.08	-0.06	-0.01
T18M - S - 8U - 2h	7.60	0.00	0.45	1.38	0.03	0.02
T18M - S - 8U - 3h	10.72	0.00	0.49	1.68	0.02	0.01
T18M - S - 8U - 4h	9.86	0.00	0.49	1.75	0.03	0.01
T18M - S - 8U - 5h	9.60	0.01	0.61	1.81	0.04	0.03
T18M - S - 8U - 6h	10.63	0.01	0.71	1.69	0.03	0.01
T18M - S - 8U - 24h	13.09	0.00	0.75	1.19	-0.11	0.02

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